

Cyanotoxin detection in freshwater systems from the North and Center of Portugal

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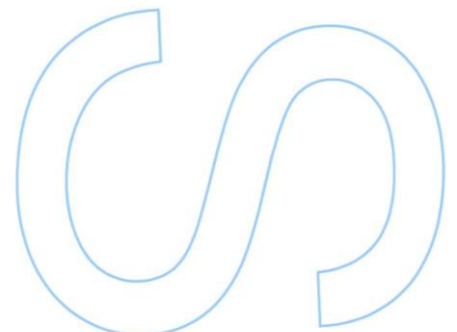
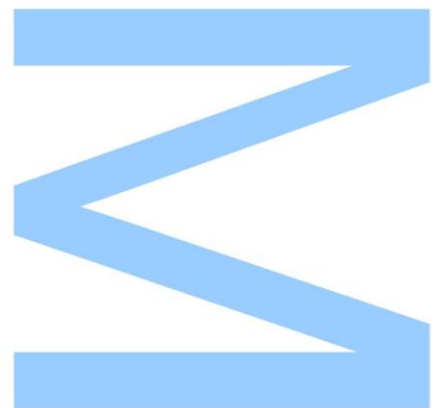
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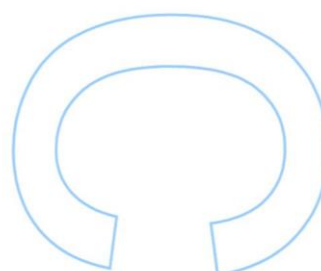
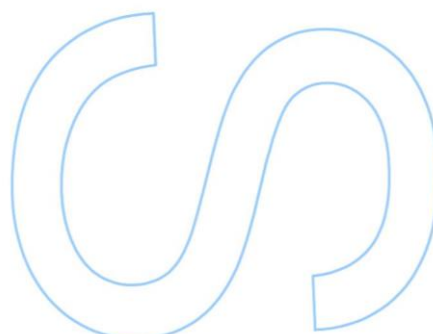
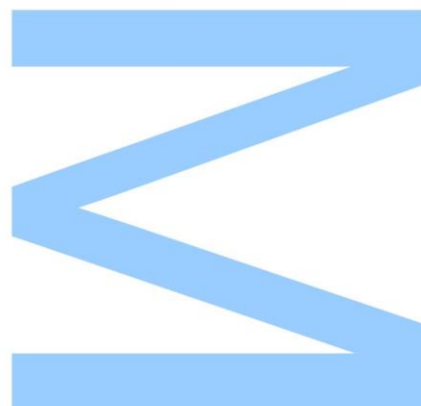




Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

In recent years, freshwater ecosystems have undergone changes in their physical, chemical and biological properties due to eutrophication processes. The increase of nutritive compounds in the water leads to an accelerated growth of all aquatic productivity, especially in the cyanobacteria communities. The densification of the cyanobacteria community in the water surface leads to the production of cyanotoxins, secondary metabolites that contribute to the degradation of the water quality and has serious consequences for both the environment, animal and human health. With a high variety of chemical variants and several degrees of toxicity, cyanotoxins can be grouped according to their mode of action in hepatotoxins (microcystins, nodularins), cytotoxins (cylindrospermopsin), neurotoxins (anatoxin-a, saxitoxin) and dermatotoxins (lyngbyatoxin, aplysiatoxin). The aim of this study is to detect the presence of microcystins, cylindrospermopsin, anatoxin-a and saxitoxin in freshwater systems from the North and Center of Portugal, through chemical, biochemical and molecular methods. To achieve such goal, environmental samples from seven freshwater systems from the North and Center of Portugal were analyzed for the presence of amplified fragments of genes involved in cyanotoxin biosynthesis (molecular methods) and, when those fragments were present, samples were quantified in order to detect and quantify toxins, through chemical and biochemical methods. The results showed the presence of microcystins, cylindrospermopsin, anatoxin-a and saxitoxin in the studied freshwater systems and the potential for their production by the cyanobacteria genera present in the water environment. Also the efficiency of the molecular methods in monitoring programs to detect the presence of cyanotoxins and their producing cyanobacteria genera revealed to be an effective and valuable tool. In the absence of other information about the presence of anatoxin-a, cylindrospermopsin and saxitoxin in Portuguese freshwater systems, this study constitutes the first report of the presence of these cyanotoxins in Portuguese freshwater systems.

Keywords: Eutrophication, Cyanobacteria, Cyanotoxins, Methods for cyanotoxin detection

Resumo

Nos últimos anos, os sistemas aquáticos têm sofrido alterações nas suas propriedades físicas, químicas e biológicas, devido aos fenómenos de eutrofização que se registam nesses mesmos sistemas. O aumento dos compostos nutritivos na água leva a um crescimento exponencial de toda a produtividade aquática, especialmente das comunidades de cianobactérias. A densificação destas comunidades nas superfícies dos recursos aquáticos leva à produção de metabolitos secundários – cianotoxinas – que contribuem ativamente para a degradação da qualidade da água e originam graves consequências a nível de saúde ambiental, animal e humana. Com uma elevada variedade de isoformas químicas e diversos graus de toxicidade, as cianotoxinas podem ser agrupadas de acordo com o seu modo de ação em hepatotoxinas (microcistinas, nodularina), citotoxinas (cilindrospermopsina), neurotoxinas (anatoxina-a, saxitoxina) e dermatotoxinas (aplisiotoxina, lingbiatoxina). Assim, o objetivo deste estudo é a deteção da presença de microcistina, cilindrospermopsina, anatoxina-a e saxitoxina em sistemas aquáticos de água doce nas regiões Norte e Centro de Portugal, através de métodos químicos, bioquímicos e moleculares. Para tal, amostras ambientais de sete pontos de amostragem das regiões Norte e Centro foram analisadas para a presença de genes envolvidos na síntese de cianotoxinas através de métodos moleculares; seguidamente, e em caso de presença desses fragmentos, as amostras foram quantificadas com recurso a métodos químicos e bioquímicos, de modo a quantificar a toxina presente no meio. Os resultados mostram a presença de microcistina, cilindrospermopsina, anatoxina-a e saxitoxina nos diversos pontos de amostragem e a presença de géneros potencialmente produtores no meio aquático. Paralelamente, a eficácia dos métodos moleculares como medida inicial de monitorização dos pontos de amostragem para a presença de cianotoxinas e respetivos géneros produtores mostrou ser bastante fiável. Na ausência de bibliografia que reporte a presença de anatoxina-a, cilindrospermopsina e saxitoxina em sistemas aquáticos portugueses, e dado os dados obtidos, este estudo apresenta-se como sendo o primeiro a relatar a presença destas cianotoxinas em sistemas de água doce Portuguesas.

Palavras-chave: Eutrofização, Cianobactérias, Cianotoxinas, Métodos de detecção de cianotoxinas

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List of Abbreviations

BMAA – β -methylanino-L-alanine

BSA – Bovine Serum Albumin

CE – Capillary Electrophoresis

CYN - Cylindrospermopsin

DNA – Deoxyribonucleic Acid

dNTPs – Deoxynucleotide Triphosphates

EDTA - Ethylenediamine Tetraacetic Acid

ELISA – Enzyme-Linked Immunosorbent Assay

FLD – Fluorescence Derivatization

HPLC – High-Performance Liquid Chromatography

HPLC-PDA – High-Performance Liquid Chromatography-Photodiode Array

IL-1 – Interleukin-1

LC-MS – Liquid Chromatography-Mass Spectrometry

LOD – Limit of Detection

MC-LR – Microcystin-LR

MS – Mass Spectrometry

NMR – Nuclear Magnetic Resonance

NRPS – Non-ribosomal Peptide Synthesis

PCR – Polymerase Chain Reaction

PDA – Photodiode Array

PKS – Polyketide Synthesis

PSP – Paralytic Shellfish Poisoning

SXT - Saxitoxin

TAE - Tris-Acetato-EDTA

TNF- α – Tumor Necrosis Factor Alpha

UV – Ultraviolet radiation

WHO – World Health Organization

1.Introduction

As a community, the evolution of human species always finds itself related to the water presence, its abundance and worldwide distribution. The exploitation of water resources has been used by humans for their locomotion, for their livelihoods and their development, whether in a technological or in a civilizational level. However, along with this development of humans, recorded mostly after the Industrial Revolution, there has also been deterioration, both in quality and biodiversity, of the available water resources exploited by man, due to the non-sustainable resource management and the constant pressures on them (Mendes and Oliveira, 2004).

The degradation of water resources reveals itself through a series of changes in their physical, chemical and biological characteristics, leading to the eutrophication of the water ecosystem, and subsequently, to several issues in terms of environmental, animal and public health.

1.1. General concepts

Widely recognized as a worldwide problem (Zhang *et al.*, 2009), the eutrophication of freshwater systems is a process characterized by increase of the nutritive compounds in the water, like nitrogen and phosphorus, leading to the accelerated growth of all aquatic biologic productivity, especially from algal communities (de Figueiredo *et al.*, 2004; Porteiro *et al.*, 2005; Nimptsch *et al.*, 2008). The state of eutrophication is determined by the observation of changes that occur in the phytoplanktonic communities, especially in the decrease of the relation between sensitive taxa/ tolerant taxa and in the global intensification of the abundance of phytoplanktonic biomass (Gonçalves *et al.*, 2006). This is a process that occurs naturally over millennia; however, this is accelerated or even triggered by human pressures exercised on freshwater systems (Porteiro *et al.*, 2005). Along with other factors, like high temperatures, physic stability of water column and inadequate agricultural procedures near water resources that leads to an excessive and unnecessary use of fertilizers (de Hoyos *et al.*, 2004; Nimptsch *et al.*, 2008), this process provides favorable conditions to the occurrence of cyanobacterial blooms, common elements of phytoplankton in most freshwater systems (Bittencourt-Oliveira, 2003; Quesada *et al.*, 2004; Papadimitiou *et al.*, 2012).

Cyanobacteria are photosynthetic prokaryotes dated from three billion years ago, commonly present in aquatic systems, especially in freshwater and marine ecosystems (Sze, 1993; de Hoyos *et al.*, 2004; Kumar *et al.*, 2011). Its presence is detected on the surface of aquatic systems in eutrophic and mesotrophic state, located in areas with a range of temperatures that allow the proliferation of cyanobacteria. The richness, in a nutrient level, of these waters, along with the suitable physical-chemical parameters, allows cyanobacteria to form dense accumulations on the water surface and then become active competitors against eukaryotic phytoplankton for the nutrients in the water and for the absorption of the active photosynthetic radiation available along the water column (Neilan *et al.*, 1999; de Hoyos *et al.*, 2004; Quesada *et al.*, 2004; Bittencourt-Oliveira *et al.*, 2010a).

One of the main consequences of this process is the production of secondary metabolites by some species of cyanobacteria, which contribute to the degradation of water quality, with severe consequences in terms of environmental, animal and human health – the cyanotoxins (Quesada *et al.*, 2004).

1.2. Cyanotoxins

Secondary metabolites with a high level of toxicity, cyanotoxins present an extended variety of chemical and molecular structures and several levels of toxicity (Kumar *et al.*, 2011). Produced by cyanobacteria, these toxins can remain inside the cell after their synthesis (intracellular) or be released to the water environment (extracellular), during cyanobacteria growth phase and/or during cellular lysis (senescence).

Given the wide variety of chemical and molecular structures, cyanotoxins can be classified at two levels: through its mode of action or only by its chemical structure. According to its chemical structure, cyanotoxins can be classified as cyclic peptides (microcystins and nodularins); alkaloids (cylindrospermopsin, anatoxin-a, anatoxin-a(S) and saxitoxin) and lipopolysaccharides (Carmichael and Liu, 2006). According to its target-organ and mode of action, cyanotoxins can be classified as hepatotoxins (microcystins, nodularins), cytotoxins (cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(S), β -methylamino-L-alanine (BMAA) and saxitoxin) and dermatotoxins (lyngbyatoxin, aplysiatoxin) (Bittencourt-Oliveira, 2003; Carmichael and Liu, 2006; Kumar *et al.*, 2011).

Cyanotoxins have the ability to accumulate in the tissues of organisms from diverse trophic levels. This accumulation in the tissues can lead to several symptoms, most of them difficult to detect and prevent and can also lead to the death of the individuals, being already responsible for high mortality rates, especially in aquatic animals (Bittencourt-Oliveira, 2003). Humans are exposed to cyanotoxins mostly through ingestion of contaminated food, especially shellfish; they can also be exposed through the accidental ingestion or contact with contaminated water during recreational activities and through the ingestion of contaminated water (Žegura *et al.*, 2011; Merel *et al.*, 2013). The most severe case already reported dates back to 1996, when 76 patients of a hemodialysis clinic in Caruaru, Brazil died due to the contact with contaminated water used in hemodialysis being the resulted symptoms described as the so called “Caruaru Syndrome” (Bittencourt-Oliveira, 2003; Bittencourt-Oliveira *et al.*, 2010b).

1.2.1. Microcystins

Microcystins comprise a group of cyclic heptapeptides whose chemical structure varies according to the amino acids in X and Z positions (Figure 1). More than 90 structural variants of microcystins are already known, despite just one or two of the isoforms dominate in each one of the producing genera (Neilan *et al.*, 1999; Bittencourt-Oliveira, 2003; Kumar *et al.*, 2011).

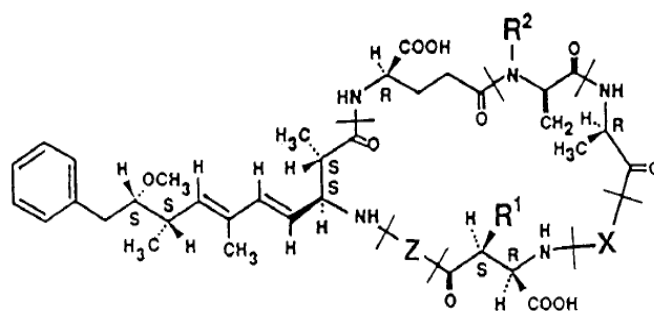


Figure 1. Chemical structure of microcystin (adapted from Neilan *et al.*, 1999).

Natural toxins produced by some species belonging to the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Planktothrix* (Xie *et al.*, 2005; Kumar *et al.*, 2011), microcystins are synthesized by a non-ribosomal enzymatic complex, which comprehends a non-ribosomal signaling pathway through an enzymatic complex peptide synthesis (NRPS) and a polyketide synthesis mechanism (PKS). This

synthesis mechanism is responsible for the incorporation of the amino acids in the peptide chain, being codified by *mcy* gene clusters (Bittencourt-Oliveira, 2003; Ouahid *et al.*, 2005; Mankiewicz-Boczek *et al.*, 2006; Bittencourt-Oliveira *et al.*, 2010).

Early detection of microcystins and their producing species leads to an effective prevention against its action. Their toxicity, as well its high stability and thermal resistance, translate into serious consequences to the environment and to biodiversity, in a direct or indirect form, through water that contains microcystins, due to their capacity to bioaccumulate over the several trophic levels of the established food chains in the affected ecosystems (Neilan *et al.*, 1999; Bittencourt-Oliveira *et al.*, 2010; Papadimitriou *et al.*, 2012).

In the human organism, microcystins act on the bile acid transport system, where occur hyperphosphorylation of the microfilaments. On hepatocytes, microcystins activate phosphorylase A2 and cyclooxygenase; in macrophages, this toxin is capable to induce tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1). Given the effects on hepatocytes and macrophages, microcystins are also considered as promoters of hepatic tumors (Neilan *et al.*, 1999; Xie *et al.*, 2005; Chen *et al.*, 2010; Papadimitriou *et al.*, 2012).

Due to its high toxicity the World Health Organization (WHO) established 0.04 μ g/kg of body weight as a daily limit of tolerable intake for microcystins and 1 μ g/L for its presence in drinking water, with the assumption that about 80% of human exposure to this toxin is due to consumption of contaminated water (Poste *et al.*, 2011). In Portugal, a guideline value for microcystins of 1 μ g/L for drinking water was established, according to the Decreto-Lei 306/2007. Despite these recommendations, cases of contamination of humans continue to be reported annually, some of them with the occurrence of deaths of individuals.

One of the most severe cases of contamination in humans reports to 1996, where 76 patients of a hemodialysis center in the city of Caruaru, Brazil, died due to the ingestion of water contaminated with microcystins and wherein the manifested disease became known as "Caruaru Syndrome" (Bittencourt-Oliveira, 2003; Bittencourt-Oliveira *et al.*, 2010b). In China, the consumption of fish originated from eutrophic lakes with a frequent record of toxic cyanobacterial blooms caused several cases of microcystin contamination through bioaccumulation processes, being already considered a serious case of public health, given the eutrophic state of most of Chinese water resources (Zhang *et al.*, 2009).

In Portugal, microcystins are present in freshwater systems, widely spread and studied across the country, being the principal cyanotoxin in Portuguese freshwater ecosystems, although without a record of human contamination due to ingestion or contact with contaminated water (Vasconcelos, 1993, 1994, 1995, 1999, 2001; Vasconcelos *et al.*, 1995, 1996; Freitas, 2009; Morais, 2009; Regueiras, 2009).

1.2.2. Cylindrospermopsin

Among the toxins with hepatotoxic, neurotoxic and cytotoxic effects (Pearson *et al.*, 2010; Neilan *et al.*, 2013), cylindrospermopsin are a group of tricyclic alkaloid toxins, with a chemical and molecular stable structure, without great variations in their amino acid composition (Aráoz *et al.*, 2010; Žegura *et al.*, 2011; Gutiérrez-Praena *et al.*, 2013). With a low molecular weight (415 Da) (Žegura *et al.*, 2011), cylindrospermopsin is a polyketide-derived alkaloid, containing a guanidine group and a hydroxide group, connected to a tricyclic-carbon skeleton (Figure 2) (Mihali *et al.*, 2008; Pearson *et al.*, 2010; Žegura *et al.*, 2011; Poniedzialek *et al.*, 2012; Gutiérrez-Praena *et al.*, 2013; Merel *et al.*, 2013). Changes in its molecular structure originate the cylindrospermopsin' analogues 7-epicylindrospermopsin, a toxic minor metabolite of *Aphanizomenon ovalisporum*, due to the occurrence of an epimer at the hydroxyl bridge, and 7-deoxy-cylindrospermopsin, given the lack of the hydroxyl group at C-7 (Mihali *et al.*, 2008).

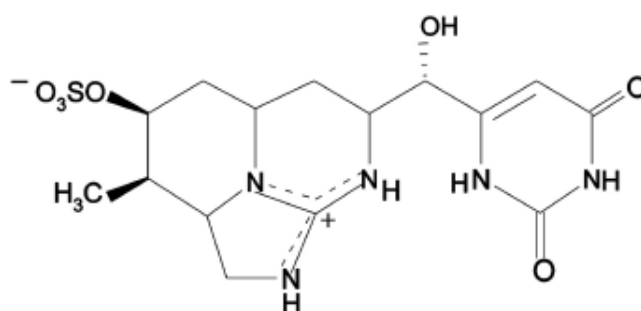


Figure 2. Chemical structure of cylindrospermopsin (adapted from van Apeldoorn *et al.*, 2007).

Produced by common species of the cyanobacteria community present in freshwater systems, like *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata*, *Lyngbya wollei*, *Anabaena bergii*, *Aphanizomenon flos-aquae* e *Anabaena lapponica* (Mihali *et al.*, 2008; Pearson *et al.*,

2010), cylindropsermopsin is a very water-soluble toxin (Žegura *et al.*, 2011), finding itself, in 90% of the total quantity of cylindrospermopsin produced by cyanobacteria, dissolved in water. The chemical structure of this toxin gives it a high stability, even in extreme environments in terms of temperature, pH and radiation, making difficult its elimination from the water (Saker *et al.*, 2004; White *et al.*, 2006; Kalaitzis *et al.*, 2010; Poniedziałek *et al.*, 2012; Gutiérrez-Praena *et al.*, 2013). Degradation of cylindrospermopsin present in the water is promoted in the presence of oxygen and inhibited in anoxic environments, wherein the adsorption process of the toxin, during sedimentation process, depends of the amount of organic carbon available (Poniedziałek *et al.*, 2012).

In recent years, exponential growth of the number of reported cases of cylindrospermopsin potentially-producing species has raised several questions regarding the impact of their presence in the environment and its capacity to bioaccumulate in the tissues of the organisms present in the affected environment (Saker *et al.*, 2004; White *et al.*, 2006). The wide geographical distribution of cylindrospermopsin and its producing species, its high solubility in water and its high presence in water in its dissolved form, causes humans to be more susceptible to cylindrospermopsin contamination, relatively to other cyanotoxins (Gutiérrez-Praena *et al.*, 2006).

In human organisms, cylindrospermopsin acts on the protein synthesis, inhibiting this process. This cyanotoxin also interferes on the metabolism of cytochrome p450 and other cellular mechanisms, therefore being classified as cytotoxic (Saker *et al.*, 2004; Aráoz *et al.*, 2009; Pearson *et al.*, 2010; Gutiérrez-Praena *et al.*, 2013). However, previous studies (Humpage *et al.*, 2000; Shen *et al.*, 2002), show that cylindrospermopsin can also be classified as carcinogenic, given the damages caused by this toxin in DNA (Deoxyribonucleic Acid) strains. At a functional level, exposure and contamination of the organism by cylindrospermopsin can cause serious damages in organs as diverse as the liver, kidneys, thymus, heart, lungs, spleen and immune system (Pearson *et al.*, 2010; Žegura *et al.*, 2011). Despite its effects in the organisms and in the environment, there are no official guidelines established by WHO for the presence of cylindrospermopsin in drinking water. However, Humpage and Falconer (2003) suggested a guideline of 1.0 µg/L, similar to the one established for microcystins. Some countries, however, have legislated the maximum quantity of cylindrospermopsin allowed in drinking water, like Queensland region in Australia (1.0 µg/L), Brazil (15 µg/L), New Zealand (1.0 µg/L) (Burch, 2008; Washington State Department of Health, 2011).

In Portugal, there are still no reports of the presence of this cyanotoxin in freshwater systems, nor an established guideline. However, a previous study (Moreira *et al.*, 2011), reported the presence of the *pks* gene in samples from Vela Lake, though, analysis by HPLC-PDA and LC-MS did not detect the presence of cylindrospermopsin in the water samples. Nevertheless, due to cylindrospermopsin's characteristics, is important to continue the monitoring of this freshwater ecosystem for the presence of the genes implicated in cylindrospermopsin biosynthesis and its production by cyanobacteria.

1.2.3. Anatoxin-a

Anatoxin-a is an alkaloid composed by a bicyclic secondary amine, being this way chemical and structurally analogue of acetylcholine, a natural neurotransmitter of the organisms (Figure 3) (Teixeira and Rosa, 2006; Yavasoglu *et al.*, 2008). Due to its chemical structure, anatoxin-a is highly unstable in natural conditions, being easily converted in non-toxic products, like dihydroanatoxin-a and epoxyanatoxin-a (Osswald *et al.*, 2007).

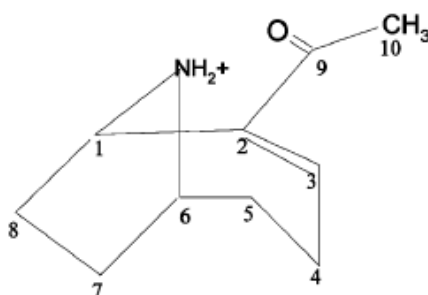


Figure 3. Chemical structure of anatoxin-a (adapted from van Apeldoorn *et al.*, 2007).

Firstly reported in 1951 in the USA, anatoxin-a was the first cyanotoxin to be chemical and functionally characterized (Osswald *et al.*, 2007; Ballot *et al.*, 2010). With low molecular weight, it possesses a high solubility in water, being easily degraded by the microorganisms present in the water and in alkaline and/or exposed to direct light environments (Teixeira and Rosa, 2006; Osswald *et al.*, 2007; Yavasoglu *et al.*, 2008). However, anatoxin-a degradation process is independent of the presence of oxygen, concluding in this way that it is not a photo-degradation process, despite it depends on direct light and high pH of the environment to accomplish (Osswald *et al.*, 2007).

Possessing an high geographical distribution, anatoxin-a is produced by phytoplanktonic and benthic species belonging to genera *Anabaena*, *Aphanizomenon*, *Cylindrospermum*, *Oscillatoria*, *Microcystis*, *Raphidiopsis*, *Planktothrix*, *Artrospira*, *Nostoc* and *Phormidium*. Its distribution, allied to its high toxicity and instability, as raised several questions about its consequences on environmental and public health (Osswald *et al.*, 2007; Ballot *et al.*, 2010; Yavasoglu *et al.*, 2010; Žegura *et al.*, 2011).

The presence of anatoxin-a in Portugal is still not reported, although previous studies (Osswald *et al.*, 2007; 2009), demonstrated that Portuguese strains of anatoxin-a producing species, present in nine freshwater systems, produces the toxin when cultivated in laboratory. So, the potential occurrence of anatoxin-a in Portuguese waters must be considered.

Anatoxin-a is an acetylcholine cholinergic agonist and mimics acetylcholine's mode of action at muscular neurotransmitters. It binds irreversibly to acetylcholine neuronal receptors, present in the neuromuscular junction, with higher affinity than acetylcholine itself (Teixeira and Rosa, 2006; Yavasoglu *et al.*, 2008; Žegura *et al.*, 2011). This connection induces the continuous stimulation of the muscle, due to the fact that anatoxin-a is not degraded by acetylcholinesterase, leading to muscular exhausting, the paralysis of the muscle and, in case of paralysis of respiratory and/or cardiac muscles, the death of the individual (Teixeira and Rosa, 2006; Žegura *et al.*, 2011).

The lack of an available treatment for cases of intoxication by anatoxin-a has led to an increasing necessity of more studies about the structure and mode of action of this cyanotoxin (Žegura *et al.*, 2011). Its chronic effects are unknown, as well as its genotoxic and carcinogenic potential (Teixeira and Rosa, 2006; Žegura *et al.*, 2011). Also, guidelines regarding the presence of anatoxin-a in drinking water are practically inexistent in most countries, including Portugal. However, New Zealand established a value of 6.0 µg/L and Washington State Department of Health suggested, in 2008, a maximum quantity of 1.0 µg/L in drinking water, due to the potent action of anatoxin-a (Burch, 2008; Washington State Department of Health, 2008).

1.2.4. Saxitoxin

Saxitoxins comprehend a group of alkaloid tetrahydro-purines which chemical and molecular structure varie according to the amino acids presents in R positions (Figure 4) (Sivonen and Jones, 1999; Llewellyn, 2006; Pearson *et al.*, 2010). Its tricyclic

structure gives saxitoxin stability and resistance to extreme conditions, like temperature and pH (Llewellyn, 2006).

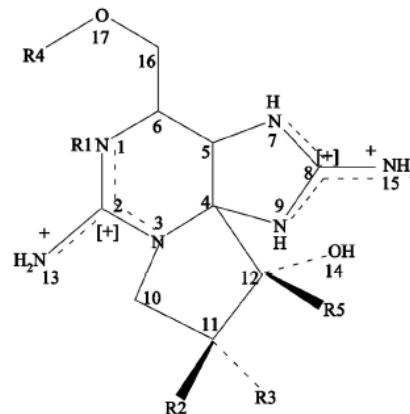


Figure 4. Chemical structure of saxitoxin (adapted from van Apeldoorn *et al.*, 2007).

Possessing over 30 different isoforms, saxitoxins are produced by marine dinoflagellates from genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* and by cyanobacteria from genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya* and *Planktothrix* (Sivonen and Jones, 1999; Landsberg, 2002; Llewellyn, 2006; Pearson *et al.*, 2010; Perreault *et al.*, 2011). The variations in the producing organisms, as well its geographical distribution, are essential factors in saxitoxin' toxic profiles, while a potentially producing genus might not produce toxic isoforms of this cyanotoxin (Pearson *et al.*, 2010).

Its high toxicity is highly harmful to organisms, wherein intoxications by contact with saxitoxins were already responsible for massive deaths of marine organisms, as well as humans (Pearson *et al.*, 2010; Perreault *et al.*, 2011). The high resistance by some organisms, like shellfish, and the ease of saxitoxin accumulation in tissues leads to an increase risk of occurrence of bioaccumulation processes which, in case of existing, affect directly all the organism in the several trophic levels of the food chain that exist in that system, creating, in this way, several environmental and public health issues (Pearson *et al.*, 2010).

Along with the consumption of contaminated water, bioaccumulation of saxitoxins through the several trophic levels is, generally, the main source of human contact with this cyanotoxin. The vast consumption of aquatic organisms, from the several levels of

the food web, makes that the occurrence of intoxications by saxitoxins are still very common worldwide (Landsberg, 2002; Perreult *et al.*, 2011).

At a human level, saxitoxins, as well as their derivatives, are the causal agents of PSP - Paralytic Shellfish Poisoning (Sivonen and Jones, 1999). This pathology characterizes itself by the blocking of muscular and nervous sodium channels, stopping this way the influx of sodium to the cells and restricting the signal transmitted between neurons. At a physiological and symptomatological level, this sodium channels block translates itself, at an initial stage, in facial numbness, vomits and diarrhea. In more acute cases, it can also cause muscular weakness, ataxia, motor incoordination, incoherence and, sometimes, respiratory insufficiency. When in lethal doses, this block causes cardiac and respiratory arrest, leading to the death of the individual (Landsberg, 2002; Pearson *et al.*, 2010). Since its discovery, numerous fatal cases of saxitoxin intoxication have been reported. Despite the knowledge of its structure, mode of action, symptomatology, there is still no cure for saxitoxin intoxication (Pearson *et al.*, 2010). Guideline values for saxitoxin are still not established by WHO, or by most of the countries. Exceptions are for New Zealand, Brazil and Australia, where the concentration of saxitoxin (expressed in SXT-equivalent) should not exceed 3.0 µg/L (Burch, 2008; Washington State Department of Health, 2011).

Saxitoxin presence in Portuguese freshwaters is still not reported, although there are reports of the presence of this cyanotoxin in coastal areas, especially in shellfish that inhabit those ecosystems (Pereira *et al.*, 2004; Martins *et al.*, 2005; Artigas *et al.*, 2007). However, it is reported the presence of saxitoxin producing species of cyanobacteria in Portuguese freshwater systems and saxitoxin production in laboratory cultures (Ferreira *et al.*, 2001).

1.3. Methods for cyanotoxin research

Cyanotoxin detection was initially made chemically in the studied water samples. Morphologic identification of producing organisms showed to be ineffective in the detection of cyanotoxin-producing species. Due to the lack of morphologic differences between producing and non-producing organisms, it is impossible to perform an initial screening of cyanotoxin-producing strains (Bittencourt-Oliveira, 2003; Kumar *et al.*, 2011).

The study, at a molecular level, of environmental samples containing cyanotoxins allows the quantification and identification of the producing strains, providing a water-monitoring program more sensitive and specific in relation to the established criteria for environmental control. Depending of the obtained sampling conditions, analysis for detection and quantification of cyanotoxins can be made using chemical, biochemical and molecular methods (Regueiras, 2009; Kumar *et al.*, 2011).

1.3.1. Chemical methods

Chemical methods are the most widely used methods to detect and quantify cyanotoxins (Moreira *et al.*, 2014). Examples of chemical techniques are High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE), Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) (Msagati *et al.*, 2006).

HPLC is most used method for cyanotoxin detection in environmental samples, along with a UV, PDA (Photodiode Array) and FLD (Fluorescence Derivatization) detectors (Moreira *et al.*, 2014). It can also be coupled with a mass spectrometer (LC-MS) for a more sensitive detection of cyanotoxins and structure elucidation (Sangolkar *et al.*, 2006). HPLC is a highly sensitive method, allowing the obtainment of qualitative and quantitative information of the cyanotoxins under study, through the analysis of two criteria: retention time and spectrum of absorption (Ouahid, 2008; Pearson and Neilan, 2008). However, despite these advantages, environmental samples have a long extraction process, because of the need to concentrate and purify the samples (Freitas, 2009). Other disadvantages of this method are the need of an expert in the field in order to conduct the analysis and interpret the obtained results; the high volume of sample needed when the toxin is in low concentrations; and the need of very expensive standards that sometimes are difficult to obtain (Rivasseau *et al.*, 1999; Rapala *et al.*, 2002; Mathys and Surholt, 2004; Sangolkar *et al.*, 2006; Pearson and Neilan, 2008; Moreira *et al.*, 2014). In comparison, ELISA is a more sensitive method than HPLC and LC-MS, with a lower LOD than HPLC and LC-MS; on the contrary, HPLC and LC-MS are more specific and selective techniques since it allows the differentiation between the several cyanotoxin isoforms (Merel *et al.*, 2013).

Liquid chromatography coupled with mass spectrometry (LC-MS) is a method more sensitive than HPLC method and therefore is widely used in cyanotoxin detection (Sangolkar *et al.*, 2006). Molecules from environmental samples are converted into ions which are subsequently detected and analyzed according to their mass charge

ratio and retention time, which limits the potential for interference in the procedure and increases the selectivity of the method (Sangolkar *et al.*, 2006; Merel *et al.*, 2013). The development of tandem mass spectrometry (MS/MS) allows the identification of the analyzed compounds through deduction of the amino acid sequence, over the fragmentation of a specific peptide (Sangolkar *et al.*, 2006).

1.3.2. Biochemical methods

The development of biochemical methods are associated with the biochemical properties possessed by cyanotoxins. Methods that use antibodies, like Enzyme-Linked Immunosorbent Assay (ELISA), Ligand-binding Assays and Enzyme Inhibition Assays, are the most commonly used in the detection and quantification of cyanotoxins (Moreira *et al.*, 2014).

Immunological ELISA tests are characterized by the use of commercial kits with mono or polyclonal antibodies in its composition, making this technique a quick and inexpensive method for the quantification of the cyanotoxins present in samples (Rivasseau *et al.*, 1999; Rapala *et al.*, 2002; Mathys and Surholt, 2004; Sangolkar *et al.*, 2006). The use of small amounts of samples, as well as its lower detection limit and its capacity to quantify samples with concentrations of toxin within the legal limits are advantages of this method when in comparison with other methods, such as HPLC (Metcalf and Codd, 2003). However, the high structural variation that occurs in cyanotoxins can cause cross-reactions between the antibodies and other variants of the toxin or other compounds present in the environmental matrix. In these cases, the results may not express, in the most accurate way, the concentration values of the studied toxin (Rapala *et al.*, 2002; Sangolkar *et al.*, 2006). Thus, and although more sensitive, ELISA tests are less selective than chemical methods, such as HPLC and LC-MS.

1.3.3. Molecular methods

Highly specific methods, molecular techniques are very useful and sensitive tools that allow the detection of a reduced number of cells (Baker *et al.*, 2002; Saker *et al.*, 2007). These methods, especially PCR (Polymerase Chain Reaction), are increasingly common in the detection of cyanotoxin potentially-producing genera, due to its low cost, its easiness in use, their rapidness and their high sensitivity (Pearson and Neilan,

2008). When the aim of the analysis is to detect cyanotoxin potentially-producing genera, it must be employed genes that are directly involved in the toxin biosynthesis (Pearson and Neilan, 2008).

Comparing bioassays with mice, microscopy and HPLC with conventional PCR, it has been demonstrated that with this molecular technique is a more effective method in monitoring cyanotoxin potentially-producer genera in water samples, thus concluding that this method can be used in the improvement of the water quality management program on the diverse aquatic ecosystems (Baker *et al.*, 2002). Saker *et al.*, (2007) also tested the effectiveness of the molecular methods on the detection of cyanotoxin potentially-producer cyanobacteria in environmental samples by comparing molecular methods with microscopic observation and chemical techniques, such as ELISA and HPLC. However, the conventional PCR method is limited by the absence of quantitative information.

1.4. Objectives

The high toxicity of cyanotoxins, as well as their impact in the environment and in the exposed organisms, creates the necessity of a specific monitoring program, designed for contaminated ecosystems, or systems with a record of regular presence of cyanobacterial blooms containing cyanotoxin potentially-producing genera. Following this necessity, the aims of this study are:

- a) Determine the ecological status of the selected ecosystems through quantification of physical and chemical parameters;
- b) Detection of the genes involved in microcystins, cylindrospermopsin, anatoxin-a and saxitoxin biosynthesis through molecular methods (PCR amplification);
- c) Quantification of the cyanotoxins present in the screened freshwater systems through biochemical methods (ELISA quantification assays);
- d) Application of chemical methods for the detection and quantification of cylindrospermopsin (HPLC-PDA and LC-MS);
- e) Evaluation of the temporal and spatial dynamics of the detected cyanotoxins in all the freshwater systems studied.

2. Materials and Methods

2.1. Study Area

2.1.1. Torrão Reservoir (Tâmega River)

Created after the construction of Torrão' hydroelectric central, in 1988, Torrão Reservoir (Figure 5) is, on the North region of Portugal, one of the main water sources for consumption and recreational activities (Oliva-Teles *et al.*, 2008; Regueiras, 2009; Torres *et al.*, 2011). Located on the Tâmega River (41°05'45.7"N, 8°15'15.4"W), the principal tributary of the Douro River in national territory, Torrão Reservoir is classified as an artificial semi-lentic freshwater system, mainly eutrophic, possessing a total water volume of 77 hm³ (Oliva-Teles *et al.*, 2008; Regueiras, 2009). Given that the system has semi-lentic characteristics and the high eutrophication of the Tâmega River and its respective hydrographic basin (Regueiras, 2009), it has been reported an increase in the frequency of toxic cyanobacterial blooms, mainly from the species *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* (Oliva-Teles *et al.*, 2008). This increase has been registered mainly in warmer months, therefore being associated with the increase of the temperature during that period (Oliva-Teles *et al.*, 2008).

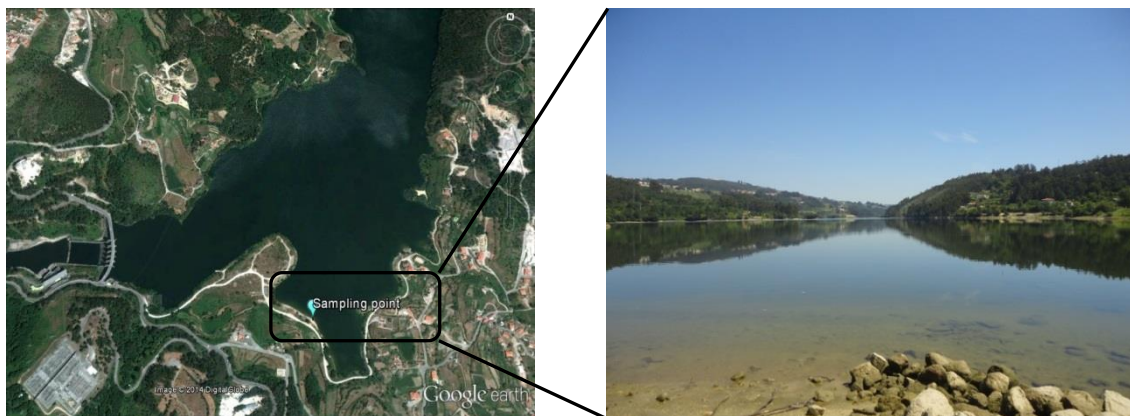


Figure 5. Location of Torrão Reservoir (image obtained using Google Earth 7.1.2.2041 software) and sampling site.

2.1.2. Parque Fluvial do Tâmega (Tâmega River)

Located on Tâmega River (41°11'45.9"N, 8°09'38.2"W), Parque Fluvial do Tâmega (Figure 6) is integrated in the reservoir created by Torrão hydroelectric central, situated about 14km downstream. Inaugurated in December of 2008, in Marco de Canaveses, Parque Fluvial do Tâmega is a recreational park, where it is allowed the practice of

aquatic activities, but people are not allowed to swim, due to the status of water. Like Torrão Reservoir, this system is an artificial semi-lentic freshwater system, classified as eutrophic, due to the hyper eutrophication of the river (Regueiras, 2009).

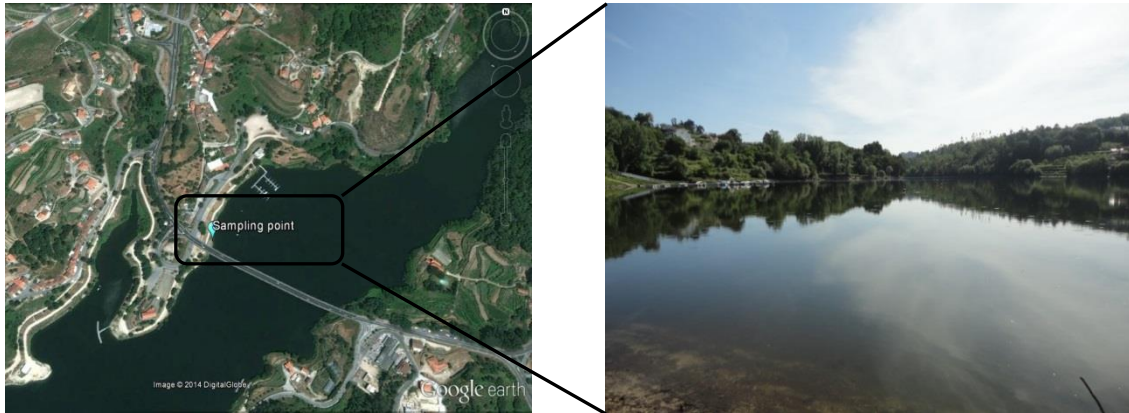


Figure 6. Location of Parque Fluvial do Tâmega (image obtained using Google Earth 7.1.2.2041 software) and sampling site.

2.1.3. Parque da Cidade do Porto

With a total area of 85 ha, Parque da Cidade is the Portugal largest urban park, composed by grassy areas, wooded areas and 4 artificial lakes. Inaugurated in 1993, Parque da Cidade is located in the middle of city of Oporto and due to its proximity to Atlantic Ocean is a stopping point for several migratory birds (Morais, 2009). Given the proximity to the sea, Parque da Cidade flora is very diverse, with the presence of 74 tree species, 42 shrub species and 10 aquatic species (Morais, 2009). In terms of natural fauna, Parque da Cidade is inhabited by swans, wild ducks, geese, fishes, frogs, rabbits, among others. In terms of cyanobacteria species presence, a previous study (Morais *et al.*, 2009) detected the presence of potentially-toxic cyanobacterial blooms, mainly from the genera *Microcystis* and *Planktothrix*. Water circulation between the lakes is made through small falls or by underground channeling. Furthermore, this water supplies the park irrigation system wherein this, in the past, was an agricultural field (Moreira, 1998; Morais, 2009).

Sampling sites of Parque da Cidade were chosen due to the connection between 3 of the 4 lakes that constituted that park (Figure 7). Situated at different highs, there is a natural water circulation from lake 1 (41°10'07.1"N, 8°40'20.5"W), in a higher position to lake 3 (41°10'01.5"N, 8°40'39.8"W), in a lower position passing, in the meantime, by lake 2 (41°10'04.5"N, 8°40'25.6"W) (Moreira, 1998; Morais, 2009).

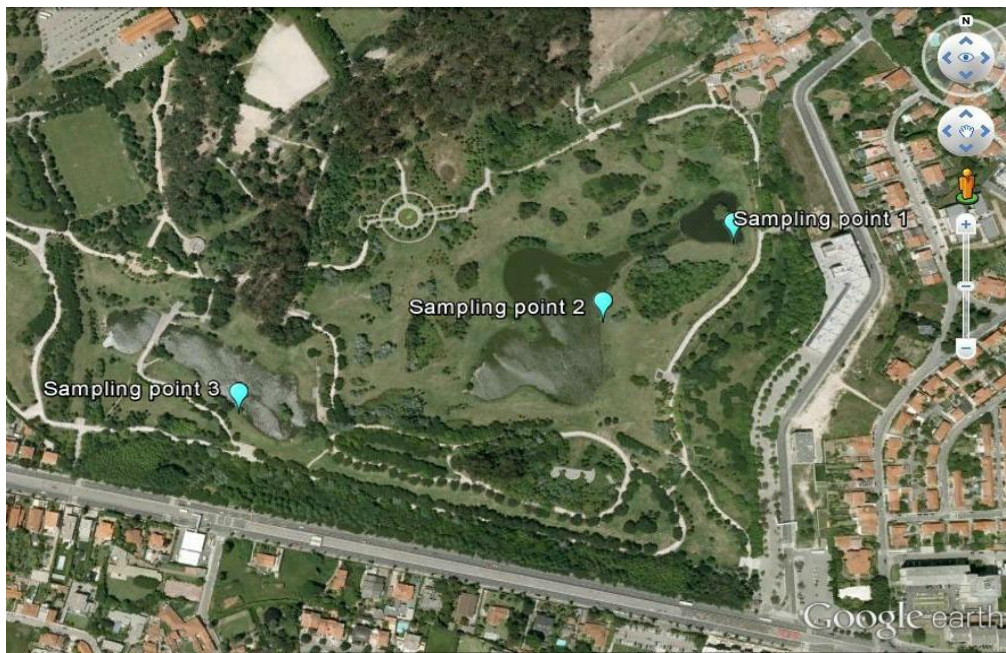


Figure 7. Location of Parque da Cidade do Porto (image obtained using Google Earth 7.1.2.2041 software). Parque da Cidade sampling sites. a) Lake 1 (sampling site 1); b) Lake 2 (sampling site 2); c) Lake 3 (sampling site 3).

2.1.4. Vela Lake

Located on the West coast of Portugal ($40^{\circ}16'23.9''\text{N}$, $8^{\circ}47'35.1''\text{W}$), Vela Lake is characterized by being a natural lentic system of low depth (mean depth: 0.9m; maximum depth: 2.4m), with a floodable surface of 70ha and a total water volume of $70 \times 10^4 \text{ cm}^3$ (Figure 8) (Castro *et al.*, 2005; Abrantes *et al.*, 2006). Given its geographical position, close to the Atlantic Ocean, and its climate, influenced by this proximity, Vela Lake is considered a place with a high conservative interest level, being actually protected by the Rede Natura 2000 program (Abrantes *et al.*, 2006). However, due to the fact that this freshwater system is surrounded, in part, by agricultural fields and areas for recreational activities, along with the sandy nature of the soil and the low depth of the lake, makes this ecosystem exposed to a high input of nutrients, leading to

the system eutrophication process (Castro *et al.*, 2005; Abrantes *et al.*, 2006). This freshwater system is characterized by the constant turbidity of the water and, on a biotic level, by frequent toxic cyanobacterial blooms, especially from *Microcystis aeruginosa* (Castro *et al.*, 2005).



Figure 8. Location of Vela Lake (image obtained using Google Earth 7.1.2.2041 software) and sampling site.

2.1.5. Mira Lake

Small lake on Portugal Central coast region (40°26'29.8"N, 8°45'07.5"W), Mira Lake is classified as a lentic system of low depth (mean depth: 2.8m; maximum depth: 3.1m), and is the result of the junction of two small streams that runs at the surface and feed the lake (Figure 9) (Gonçalves *et al.*, 1996; Castro *et al.*, 2005). Possessing a surrounding area composed essentially by agricultural fields, this lake is exposed to a high quantity of nutrients coming from the streams that feed the lake and that cross this fields (Castro *et al.*, 2005). However, urban areas that are located in the North-West side of this freshwater system make that Mira Lake also is exposed to anthropogenic pressures resultant from recreational activities, as fishing and aquatic sports, as well as the effluents that result from urban wastes originated in that same areas (Gonçalves *et al.*, 1996; Castro *et al.*, 2005; Freitas, 2009). This way, and given the pressures suffered, Mira Lake is actually classified as an eutrophized freshwater system (Castro *et al.*, 2005). At a biotic level, this freshwater system has a low cover of macrophytes (Castro *et al.*, 2005) however, according to Vasconcelos *et al.* (1993) this system has frequent reports of cyanobacterial blooms, mostly from *Microcystis aeruginosa*.

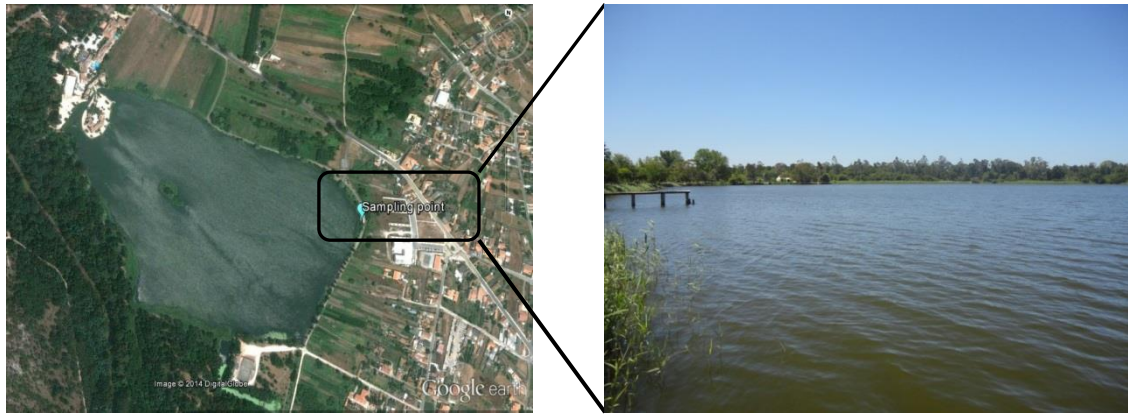


Figure 9. Location of Mira Lake (image obtained using Google Earth 7.1.2.2041 software) and sampling site.

2.2. Sample collection and treatment

Sample collection was performed from 2012 to 2013, monthly, from May to September in 2012 and from May to October in 2013. This period was chosen according to previous studies regarding ideal conditions for cyanobacterial blooms and cyanotoxin production (Vasconcelos, 1994; Regueiras, 2009).

In all the sampling sites, pH of the water was measured, using a WTW Multiline P3 meter (WTW, Germany). Additionally, atmospheric temperature values were required to IPMA, the Portuguese Institute for Sea and Atmosphere.

So, for temperature data from Torrão Reservoir and Parque Fluvial do Tâmega, it was provided data from Luzim weather station (41°09'N, 8°14'W); for temperature values for Parque da Cidade, data were provided from Porto/Pedras Rubras weather station (41°14'N, 8°39'W); for data from Vela Lake, temperature values from Figueira da Foz weather station (40°09'N, 8°51'W) were provided and for Mira Lake, temperature data were provided from Dunas de Mira weather station (40°25'N, 8°44'W).

Collected water volumes were between 2500 mL and 5000 mL according to the sampling site. Larger sampling sites (Torrão Reservoir, Parque Fluvial do Tâmega, Mira and Vela Lakes) required larger volumes to be collected while smaller sampling sites (Parque da Cidade Lakes) require smaller volumes to be collected. Water samples were collected using plastic bottles previously sterilized and kept under refrigerated conditions until arrival at the laboratory. Simultaneously, 15 mL of water for

ELISA quantification assays were collected at each site using sterilized falcon tubes that were also transported to the laboratory under the same refrigerated conditions.

2.3. Molecular analysis

2.3.1. DNA extraction

Collected water samples were processed within a period of 24 hours after their collection. The samples were filtered, with a system of vacuum filtration, using sterile Munktell MGC micro-glass fiber paper filters, with a porosity of 1.2 μm and a diameter of 47mm. After filtration, the water was discarded and the filters were stored at -20°C until posterior molecular analysis. For DNA extraction the filters content was scraped with a sterile blade and placed into a sterile Eppendorf tube.

Genomic DNA from the samples was extracted with PureLink™ Genomic DNA Mini Kit (Invitrogen, CA, USA) extraction kit, according to the manufacturer's protocol for Gram-negative bacteria, with some changes in reagents volumes. Due to high quantity of biomass collected from the filters, digestion buffer and proteinase K volumes were triplicated, to submerge the total of the sample during cellular lysis process. For this proceeding, it was used a centrifuge Eppendorf Centrifuge 5415R and a thermomixer plate Eppendorf Thermomixer compact. For the elution step, it was established a volume of 50 μL of elution solution for each DNA sample.

The presence and integrity of total genomic DNA were confirmed using 1,0% agarose gel electrophoresis (Invitrogen UltraPure™ Agarose - Invitrogen, CA, USA) at 1%, stained with a solution of ethidium bromide (10mg/mL - BioRad, CA, USA), in a 1x Tris-Acetate EDTA buffer (Invitrogen UltraPure™ 10x TAE Buffer – 400mM Tris-Acetate, 10mM EDTA, pH 8.3 \pm 0.10). For electrophoresis, it was applied a constant voltage of 100V, during 30 min. In gel wells, 10 μL of each DNA sample, along with 1 μL of 1x gel loading buffer (Nucleic acid sample loading buffer 5x, BioRad – 50mM Tris-HCL, pH 8, 25% Glicerol, 5mM EDTA, 0.2% Bromophenol Blue and 0.2% Xylene FF) were loaded. Total genomic DNA was visualized and photographed with a transilluminator CSL-MICRODOC System, under UV light, coupled with a Cannon PowerShot G9 camera system.

2.3.2. PCR amplification

For this experiment, the PCR amplifications were made using Promega GoTaq® Flexi DNA Polymerase (Promega, WI, USA) reagents and in all the PCR reactions was used a final volume of 20µL. Each reaction contained 4µL of 5x Green GoTaq® Flexi Buffer, 2µL of 25mM MgCl₂ solution, 2µL of Primer forward, 2µL of Primer reverse, 1µL of 2.5 mM dNTP's mix, 0.5µL of BSA (Bovine Serum Albumin) (10mg/mL), 0.1µL of GoTaq® DNA Polymerase (5u/µL) and 1µL of DNA. The reactions occurred on one of these thermocyclers: Biometra Professional Thermocycler (Biometra, Germany) and BioRad MyCycler™ Thermal Cycler (BioRad, CA, USA).

To confirm the presence of cyanobacterial DNA in all the extracted samples, it was performed the amplification of the 16S rRNA gene, specific for the presence of cyanobacterial DNA, through the use of the set of primers 27F/809R (Table 1). Amplification program included an initial denaturation step of 92°C for 2 min, followed by 35 cycles with one cycle consisting of a DNA denaturation step at 92°C for 20s; an annealing step at 50°C for 30s and an extension step at 72°C for 60s.

PCR products were stored at -4°C until electrophoresis. In agarose gel electrophoresis it was used the same conditions as were for the visualization of the total genomic DNA. DNA ladder used was 1kb Plus DNA Ladder (fragments from 100bp to 12kb) (Invitrogen, CA, USA).

After confirmation of the positive results obtained with 16S rRNA amplification, fragments of the genes implied in cyanotoxin synthesis were amplified. For the analysis of the fragments corresponding to microcystin synthesis the primer sets for the *mcy* gene cluster *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE* and *mcyG* were used (Table 1). In the amplification of *mcyA* gene cluster, it was used the following program: an initial denaturation step at 95°C for 2 min, followed by 35 cycles with one cycle consisting of a denaturation step at 95°C for 90s; an annealing step at 56°C for 30s and an extension step at 72°C for 50s. For amplification of gene clusters *mcyB*, *mcyC*, *mcyD*, *mcyE* and *mcyG*, the program used included an initial denaturation step at 94°C for 5 min and 35 cycles consisting in 3 steps: a denaturation step at 95°C for 60s; an annealing step at 52°C for 30s and an extension step at 72°C for 60s. A positive control used in the amplifications of the *mcy* gene markers was previously extracted from a pure culture of a *Microcystis aeruginosa* strain LEGE 00063 and as a negative control was used sterile water.

Table 1. Primer sets used in the PCR amplifications.

<i>Gene</i>	<i>Primer</i>	<i>Primer sequence 5'-3'</i>	<i>Fragment</i>	<i>Reference</i>
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	780	Neilan <i>et al.</i> , 1997
	809R	GCTTCGGCACGGCTCGGGTCGATA		Jungblut <i>et al.</i> , 2005
<i>mcyA</i>	CD1F	AAAATTTAAAGCCGTATCAAA	297	Hsbergues <i>et al.</i> , 2003
	CD1R	AAAAGTGTTTTATTAGCGGCTCAT		
<i>mcyB</i>	2156-F	ATCACTTCAATCTAACGACT	955	Mikalsen <i>et al.</i> 2003
	3111-R	AGTTGCTGCTGTAAGAAA		
<i>mcyC</i>	PSCF1	GCAACATCCCAAGAGCAAAG	674	Ouahid <i>et al.</i> 2005
	PSCR1	CCGACAACATCACAAGGC		
<i>mcyD</i>	PKDF1	GACGCTCAAATGATGAAAC	647	Ouahid <i>et al.</i> , 2005
	PKDR1	GCAACCGATAAAAACTCCC		
<i>mcyE</i>	PKEF1	CGCAAACCCGATTTACAG	755	Ouahid <i>et al.</i> , 2005
	PKER1	CCCCTACCATCTTCATCTTC		
<i>mcyG</i>	PKGf1	ACTCTCAAGTTATCTCCCTC	425	Ouahid <i>et al.</i> , 2005
	PKGR1	AATCGCTAAAACGCCACC		
AMT	AMT Fw	ATTGTAAATAGCTGGAATGAGTGG	1105	Kellmann <i>et al.</i> , 2006
	AMT Rev	TTAGGGAAGTAATCTTCACAG		
<i>pks</i>	K18	CCTCGCACATAGCCATTGTC	422	Fergusson <i>et al.</i> , 2003
	M4	GAAGCTCTGGAATCCGGTAA		Schembri <i>et al.</i> , 2001
<i>pks</i>	M4	GAAGCTCTGGAATCCGGTAA	650	Schembri <i>et al.</i> , 2001
	M5	AATCCTTACGGGATCCGGTGC		
<i>ps</i>	M13	GGCAAATTGTGATAGCCACGAGC	597	Schembri <i>et al.</i> , 2001
	M14	GATGGAACATCGCTCACTGGTG		
<i>anaC</i>	anaC-genF	TCTGGTATTCACTCCCCTCTAT	366	Rantala-Yilmén <i>et al.</i> , 2011
	anaC-genR	CCCAATAGCCTGTCATCAA		
<i>Anabaena</i> <i>anaC</i>	anaC-anabF	GCCCGATATTGAAACAAGT	263	Rantala-Yilmén <i>et al.</i> , 2011
	anaC-anabR	CACCCTCTGGAGATTGTTTA		
<i>sxt</i>	sxtI 682F	GGATCTCAAAGAAGATGGCA	195	Lopes <i>et al.</i> , 2006
	sxtI 877R	GCCAAACGCAGTACCACTT		

For identification of the genes responsible for cylindrospermopsin biosynthesis, the primer sets M13/M14 (*ps* – Peptide Synthesis), M4/M5 (*pks* – Polyketide Synthase), K18/M4 (*pks*) and AMT Fw/AMT Rev (AMT - Aminomethyltransferase) were used

(Table 1). The amplification scheme used for M13/M14, M4/M5 and K18/M4 primer sets was: an initial denaturation step at 95°C for 2 min, followed by 35 cycles, in which one cycle consists of a denaturation step at 95°C for 90s; an annealing step at 55°C for 30s and an extension step at 72°C for 50s. Amplification of AMT primer set followed this scheme: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles, consisting in 3 steps: a denaturation step at 94°C for 10s; an annealing step at 50°C for 20s and an extension step at 72°C for 60s. As a positive control for the PCR amplifications of *ps*, *pks* and AMT gene markers, an extract from a pure culture of a *Cylindrospermopsis raciborskii* strain LEGE 97047 was used and sterile water was used as a negative control.

Detection of genes involved in anatoxin-a synthesis was made through the amplification of *anaC* gene. In this amplification, the primer sets *anaC*-gen, for general amplification, and *anaC*-anab, specific for the genus *Anabaena* were used (Table 1). Amplification scheme for this 2 primer sets consisted of an initial denaturation step at 94°C for 2 min and 35 cycles, in which one cycle consists in 3 steps: a denaturation step at 94°C for 30s; an annealing step at 58°C for 30s for *anaC*-gen primers and at 60°C for 30s for *anaC*-anab primers; and an extension step at 72 °C for 30s. An extract from a pure culture of an *Anabaena* sp. strain LEGE X-002 was used as a positive control and sterile water was used as a negative control for both primer sets.

For detection of the genes implicated in saxitoxin biosynthesis, *sxtI* 682F/877R primers were used for PCR amplification. The amplification protocol used for this primer set included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation step at 94 °C for 10s, an annealing step at 52 °C for 20s and an extension step at 72 °C for 60s. As a positive control an extract from a pure culture of an *Aphanizomenon gracile* strain LMECYA 040 was used and as a negative control was used sterile water.

The presence of all the amplified fragments was confirmed with an agarose gel electrophoresis at 1.5%, in the same conditions applied in the visualization of the total genomic DNA. In gel wells, it was loaded 10µL of PCR amplification product.

2.4. Immunological assays (ELISA tests)

2.4.1. Sample preparation

Samples (15 mL of water on sterile falcons) that were stored at -20 °C after sampling were defrosted. Afterwards they were sonicated, on maximum power (60Hz) during 5 min (on ice), using an ultrasound processor (Vibra Cell™ – Sonics & Materials Inc., Danbury, CT, USA.). For anatoxin-a tests, samples were treated under minimum light conditions, giving the molecule an increased stability. Later, 1.5mL of the sonicated sample was filtered using 0.45µm Whatman™ filters. Until the realization of all the ELISA tests, the samples were stored at -20 °C.

2.4.2. ELISA quantification assays

ELISA quantification assays were done through the use of available commercial kits for the detection of the four cyanotoxins under this study: microcystins, cylindrospermopsin, anatoxin-a and saxitoxin. All the samples that were given positive results in the molecular analysis were used for quantification with this assay. The analysis of the obtained results was made using Microsoft™ Office Excel, performing the calculations provided by manufacturers.

Microcystins

Microcystins quantification was performed using Microcystins/Nodularins ADDA ELISA kit (Microtiter Plate) (Abraxis, PA, USA). This test is based on the recognition of microcystins, nodularins and their isoforms with the use of specific antibodies. With this test, it is impossible to determine which microcystin congener is being produced so the obtained values are expressed as the total quantity of microcystin-LR equivalent present in the water sample.

The calibration for this test was made using standards at 0.15, 0.40, 1.0, 2.0, 5.0 µg/L levels and with a control at 0.75 ± 0.185 µg/L. The protocol was performed according to the manufacturer's protocol using duplicated replicates. Microcystins present in the samples will compete with a Microcystin-protein analogue present in the plate for the binding sites of the antibodies present in the solution. Then, and after wash, it is added a second antibody-HRP label to the plate, which will again compete

with the microcystins present in the sample. Since each well of the kit has the same amount of binding sites and each well receives the same quantity of antibodies that compete with microcystins, the intensity of the blue color produced is inversely proportional to the quantity of microcystins in the sample, due to the fact that the blue color is more intense when there is a higher connection of antibodies-HRP label with the binding sites of the plate.

The obtained results were analyzed with the aid of a Synergy HT spectrophotometer (BioTek, VT, USA) at 450 nm of wavelength measurement and expressed in $\mu\text{g/L}$. Samples with quantification values above $5\mu\text{g/L}$ were diluted in a proportion of 1:10 to calculate, in a more accurate way, the quantity of microcystins; samples with values below the detection limit – $0.10\ \mu\text{g/L}$ - were considered as negative.

Cylindrospermopsin

Cylindrospermopsin quantification by ELISA was only performed in Vela Lake samples, due to previous studies that showed the possibility of cylindrospermopsin's production by the existing cyanobacterial genera, in this freshwater system Cylindrospermopsin ELISA (Microtiter Plate) kit (Abraxis, PA, USA) was used to quantify total amount of cylindrospermopsin present in the water samples. This test is based on the recognition of cylindrospermopsin by specific antibodies and when present in a sample, cylindrospermopsin will compete with cylindrospermopsin-HRP antibodies for the binding sites of rabbit anti-cylindrospermopsin antibodies in solution. After that, the anti-cylindrospermopsin antibodies are bound by a second antibody (goat anti-rabbit) present in the wells of the plate. The blue color generated after the wash of the plate is inversely proportional to the concentration of cylindrospermopsin of the tested samples.

The calibration for this test was made through the use of standards composed by small amounts of cylindrospermopsin, at 0.05, 0.10, 0.25, 0.50, 1.0 and $2.0\ \mu\text{g/L}$ and a control at $0.75 \pm 0.15\ \mu\text{g/L}$, despite the detection limit for this test is situated in $0.040\ \mu\text{g/L}$. The protocol was performed according to the manufacturer's instructions using triplicate replicates. The results, expressed in $\mu\text{g/L}$ were analyzed using a Synergy HT spectrophotometer (BioTek, VT, USA), at 450 nm wavelength measurement. Samples with quantification values below the detection limit – $0.040\mu\text{g/L}$ – were considered as negative.

Anatoxin-a

Quantification of anatoxin-a by ELISA was performed using the Anatoxin-a Receptor-Binding Assay (Microtiter Plate) (Abraxis, PA, USA) kit, where it was determined the total quantity of anatoxin-a present in the samples, through the affinity of anatoxin-a for nicotinic acetylcholine receptors (nAChRs). When present in a sample, anatoxin-a will compete with the biotinylated alpha-bungarotoxin for the acetylcholine binding sites of nAChRs present in the plate. After the wash of the plate, a streptavidin-HRP solution is added so it is possible for the colorimetric detection of the biotinylated alpha-bungarotoxin bound on the receptor. The generated blue color in this test is inversely proportional to the concentration of anatoxin-a in the tested samples.

Calibration for this test is made through the use of standards that contain small amounts of anatoxin-a at levels of 10, 35, 125 and 500 µg/L, being 10 µg/L the limit of detection for this kit. The protocol was performed according to the manufacturer's protocol using triplicate replicates and with minimum exposure to direct light, given the high instability of the toxin when in presence of light. A Synergy HT spectrophotometer (BioTek, VT, USA) was used to analyze the samples at 450 nm of wavelength measurement, and the results were expressed at µg/L. Quantification values for anatoxin-a below the detection limit (10 µg/L) were considered as negative.

Saxitoxin

Saxitoxin quantification by ELISA was done through the use of the commercial kit Saxitoxin (PSP) ELISA, Microtiter Plate (Abraxis, PA, USA). This test is based in the recognition of saxitoxin through specific antibodies. The presence of saxitoxin is detected due to the direct competition of the saxitoxin present in the sample with the saxitoxin-enzyme conjugate for the binding sites of rabbit anti-saxitoxin antibodies in solution. Then, the saxitoxin antibodies are bound by a second antibody (anti-rabbit) present in the plate wells. The blue color generated from this process is inversely proportional to the concentration of saxitoxin present in the tested samples.

The calibration of this kit is made through standards that contain small amounts of the toxin at levels of 0.02, 0.05, 0.1, 0.2 and 0.4 µg/L, being 0.015 µg/L the detection limit for this toxin. The protocol was performed according to the manufacturer's protocol using triplicate replicates. The results, expressed in µg/L, were analyzed with the aid of

a spectrophotometer Synergy HT (BioTek, VT, USA) at 450 nm wavelength measurement. Quantification values below 0.015 µg/L were considered as negative.

2.5. Chemical analysis

2.5.1. Sample preparation

Collected water samples from Vela Lake were filtered through a vacuum filtration system, using Munktell MGC micro-glass fiber paper filters, with a porosity of 1.2 µm and a diameter of 47 mm (Munktell, Germany). HPLC-PDA analysis was performed with both particulated and dissolved matter, in order to quantify the presence of cylindrospermopsin in biomass cells and the media. Samples extracted from July and August 2012 were only from filter content, given the impossibility to lyophilized the resulting filter water. Filters were placed in a beaker and 15 mL of a H₂O+0.1% TFA (trifluoroacetic acid) solution added, then it was crushed mechanically until the obtaining of a homogeneous solution. After that, the solution was submitted to ultrasounds (Vibra Cell™ – Sonics & Materials Inc., Danbury, CT, USA), in order to lyse the remaining cells in the solution and release its cellular content. Samples were centrifuged in a Sorvall RT Legend Benchtop Centrifuge (Thermo Scientific, MA, USA) at a velocity of 4995 g for 10 min at 4°C and supernatant was collected. Samples were kept overnight at 4°C and submitted again to the extraction process. After centrifugation, supernatant was collected from 2012 samples and submitted to a solid phase extraction (SPE) process, in order to purify and concentrate the samples. Supernatants collected from 2013 samples were collected and evaporated completely in a rotor evaporator (Büchi, Switzerland) at low temperature (<35°C) and low pressure. SPE for 2013 samples was not performed, since it was determined by HPLC-PDA that for this matrix was only necessary to concentrate the sample. Samples were resuspended in a solution of H₂O+0.1% TFA in volumes of 300 µL to 500 µL, depending on the amount of sample concentrated and placed in vials for posterior analysis. For lyophilized samples extraction process, 1500 to 2000 mL of filtered water was stored at -80°C and then lyophilized. The resulting lyophilized material was then weighted and submitted to the same extraction process detailed previously for the particulate matter.

2.5.2. Detection and quantification by HPLC-PDA

Samples were quantified in a HPLC system coupled with a photodiode array (PDA) detector Waters Alliance 2695 (Waters, MA, USA), containing a reverse phase column Lichrospher C18 (250 mm x 4.6 mm i.d., 5 μ m) (Merck, Lisbon, Portugal) maintained at 40°C and using a scanning between 210 and 400 nm with a fixed wavelength at 262 nm. Isocratic elution used was 5% of methanol from 2 solvents: 1) Mili-Q ultrapure water acidified with 0.1% (v/v) of TFA and 2) 100% methanol acidified with 0.1% (v/v) TFA with a flux of 0.9 mL/min and an injection volume of 10 μ L. All solvents used in HPLC system were filtered with GH Polypro (GHP) Membrane Disk Filters, with a porosity of 0.2 μ m and 47 mm of diameter (Pall, NY, USA). A Cylindrospermopsin (CYN) standard (0.5, 1, 2, 5, 10, 20 and 25 μ g/mL) diluted in Mili-Q ultrapure water was used to calibrate the HPLC system ($y=13435x+885.67$ $R^2=0.9998$). Empower 2 Chromatography Data (Waters, MA, USA) software was used in order to obtain cylindrospermopsin concentration in samples. The limit of detection (LOD) for cylindrospermopsin in biomass was 0.3 μ g/mL, based on a signal-to-noise ratio of 3 ($S/N \geq 3$), wherein the limit of quantification (LOQ) for CYN was 0.8 μ g/mL, based on a signal-to-noise ratio of 10 ($S/N \geq 10$).

2.5.3. Detection by LC-MS

LC-MS analysis was performed in order to confirm the presence or absence of cylindrospermopsin in environmental samples from Vela Lake previously analyzed by HPLC-PDA. Samples for LC-MS analysis were in a solution of water acidified with 0.1% of TFA.

Samples collected in 2012 were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled with Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA), with a column Hypersil GOLD (100 x 4.6 mm i.d., 5 μ m) (Thermo Scientific, MA, USA). An isocratic elution method was used and the solvents were: 1) formic acid/ultrapure water Mili-Q at 0.1% (v/v) and 2) formic acid/methanol at 0.1% (v/v). The injection volume was 10 μ L and a flux of 0.8 mL/min was used. Cylindrospermopsin was analyzed by its precursor ion $[M + H]^+$ m/z 415.6 and confirmed by the presence of

characteristic fragment ions m/z produced for an collision energy of 25. Collected data was interpreted using Xcalibur™ software.

2.6. Statistical analysis

Maximum and minimum atmospheric temperature data was initially tested to determine the normal distribution of the data using the Shapiro-Wilk normality test and for homogeneity of variance using the Levene test. Statistical significance was inferred at $p \leq 0.05$. Statistical significant differences of the maximum and minimum temperatures between the North and Center regions of Portugal were calculated with the use of a Student's t-test for Independent Samples. Statistical analysis of data was performed using IBM SPSS software version 22 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Physical/Chemical parameters

3.1.1. Temperature

Temperature data from sampling sites on the dates of collection were required to IPMA.

Statistical analysis of the collected data was performed, in order to conclude about the statistical significance of the same. Given the data obtained, it was analyzed the significance of maximum and minimum temperature in each geographical region.

North Region

Graphically, it is possible to conclude that, in general, temperatures in 2013 sampling period were higher than the ones registered in 2012, with exception for May, where both minimum and maximum temperatures were higher in 2012 than in 2013 (Figures 10 and 11) and for August, where maximum temperatures in 2012 registered values above the ones recorded in 2013 (Figures 10 and 11). Statistical analysis of the obtained data showed no significant differences in both maximum temperatures ($p=0.735$) and minimum temperatures ($p=0.679$) in 2012 and 2013 in both weather stations.

Temperature values in Luzim weather station

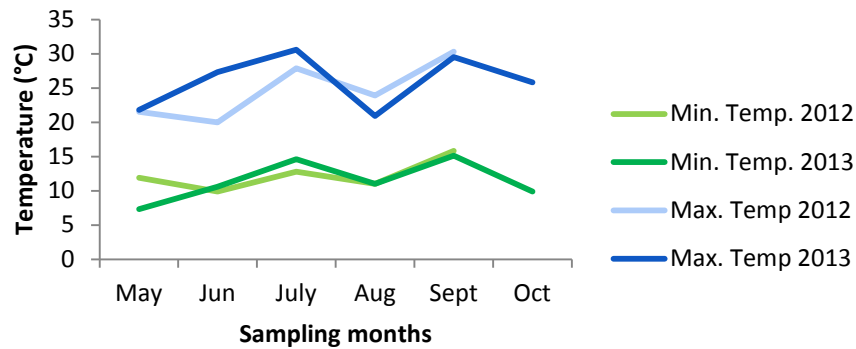


Figure 10. Minimum and maximum temperatures registered in Luzim weather station in the sampling periods of 2012 and 2013 (data provided by IPMA).

Temperature values in Porto weather station

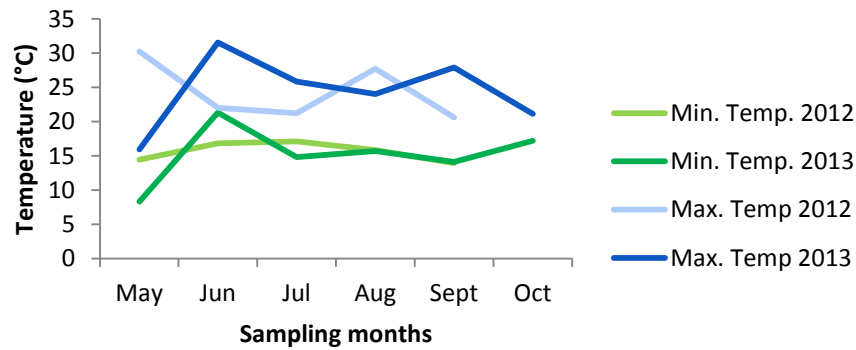


Figure 11. Minimum and maximum temperatures registered in Porto weather station in the sampling periods of 2012 and 2013 (data provided by IPMA).

Center Region

After observation of the figures below (Figures 12 and 13), it is possible to conclude that, in general, both maximum and minimum temperatures were higher in 2013 than in 2012, except in September, where the minimum temperature value in both stations were higher in 2012 rather than in 2013 and May in Mira station, where both maximum and minimum values in 2012 were above the ones registered in 2013. Despite this differences, statistical analysis showed no significant differences in both maximum ($p=0.114$) and minimum ($p=0.225$) temperatures in 2012 and 2013 in both weather stations.

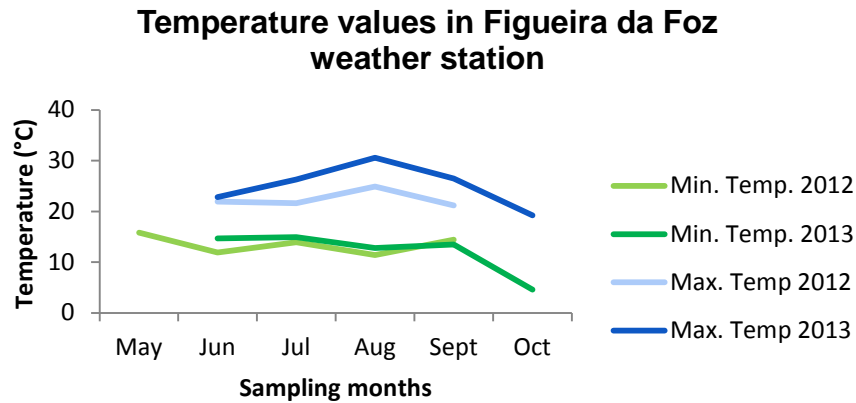


Figure 12. Minimum and maximum temperatures registered in Figueira da Foz weather station in the sampling periods of 2012 and 2013 (data provided by IPMA).

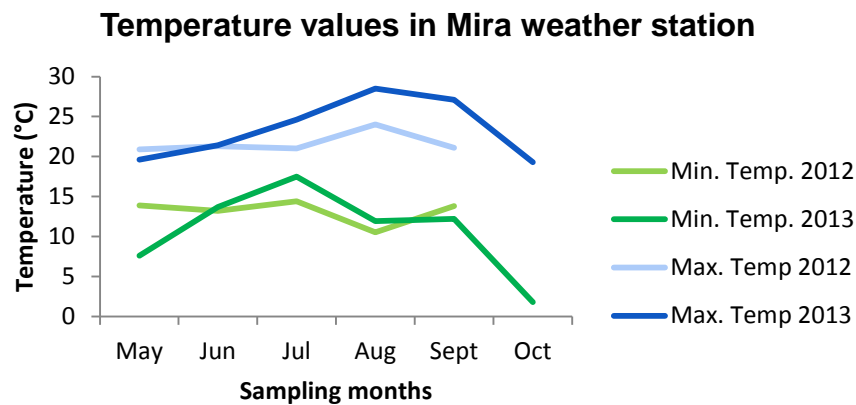


Figure 13. Minimum and maximum temperatures registered in Mira weather station in the sampling periods of 2012 and 2013 (data provided by IPMA).

3.1.2. pH

In sampling sites, monthly sampling was made between May and September in 2012, and May and October in 2013. pH measure went made *in situ*, using a pH meter. pH values were generally higher in the 2012 sampling period than in the same period in 2013, with some exceptions specially in August and September.

Torrão Reservoir

In Figure 14, it is possible to observe a variation in the registered pH values in the first three months of each sampling period. Comparing the two sampling periods, it is observable that the variation of the pH values registered in May, June and July of 2013

is consistent with the variation of the pH values from May, June and July of the 2012 sampling period, although the pH values in 2013 are lower than the ones registered in 2012. However, given the lack of data in August, September and October of 2012, it is not possible to establish a direct relation between the two sampling periods, regarding the variations in the pH of the freshwater system.

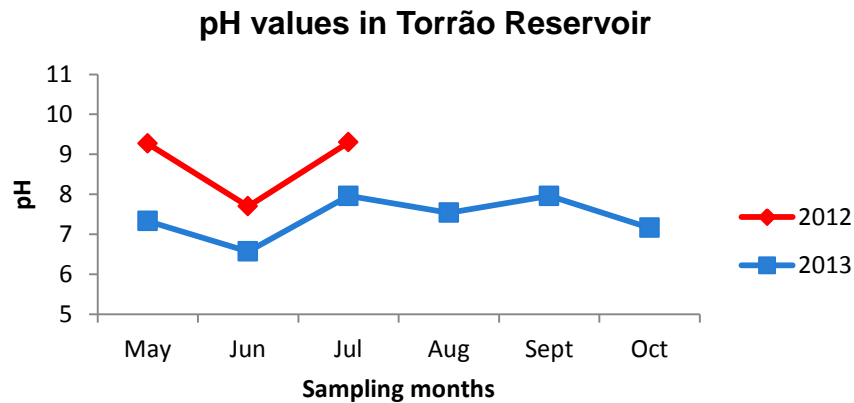


Figure 14. pH values registered in Torrão Reservoir in the sampling periods of 2012 and 2013.

Parque Fluvial do Tâmega

In this sampling site, it is possible to verify a seasonal variation within each sampling period, and between sampling periods. It is also possible to verify that the initial months of each sampling points possess lower pH values, by 2 values, relatively to the values recorded in summer months (July and August). In 2012, it is observable that the pH values registered in September stabilized with the ones recorded in July and August, contrary to the values recorded for September 2013, in which is recorded an abrupt drop in the pH values registered.

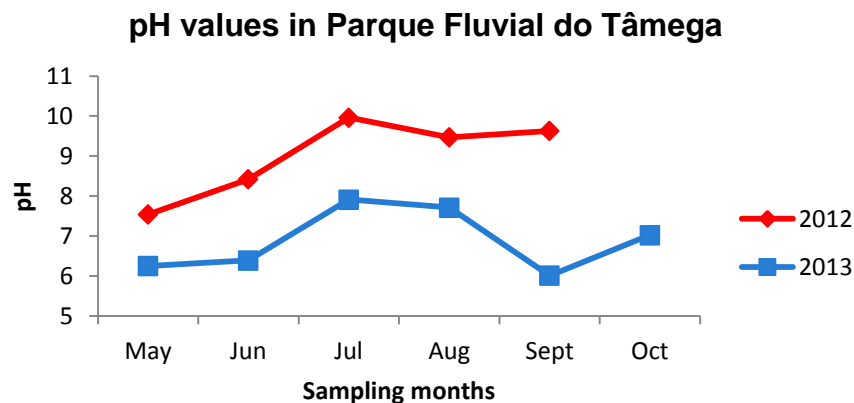


Figure 15. pH values registered in Parque Fluvial do Tâmega in the sampling periods of 2012 and 2013.

Parque da Cidade do Porto Lake 1

After observation of the figure below (Figure 16), it is not possible to establish a seasonal variation of the pH values, comparing the 2 sampling periods.. In 2012, high pH values were initially recorded which tend to decline with the advance of the sampling period, with exception for September, that register values similar to the ones registered in June; in 2013, it is observed that there is a consistency in the pH values registered, with exception for June and September, where is registered a sudden drop in the pH of water and a sudden rise in the pH of the water system, respectively.

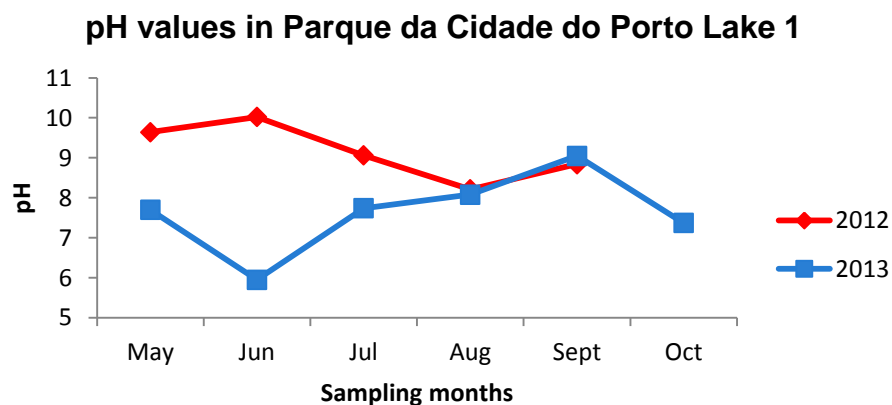


Figure 16. pH values registered in Parque da Cidade do Porto Lake 1 in the sampling periods of 2012 and 2013.

Parque da Cidade do Porto Lake 2

In this sampling point, it is not possible to observe the existence of a pattern of seasonal variability, comparing the 2 years of sampling (Figure 17). While in 2012 is registered a rise in the pH values and then a subtle decrease of the same, in 2013 is recorded a fall in the pH values registered in July, compared with the ones obtained in May and June and then, a rise of the values in August and September to values above the ones recorded in the beginning of the sampling period.

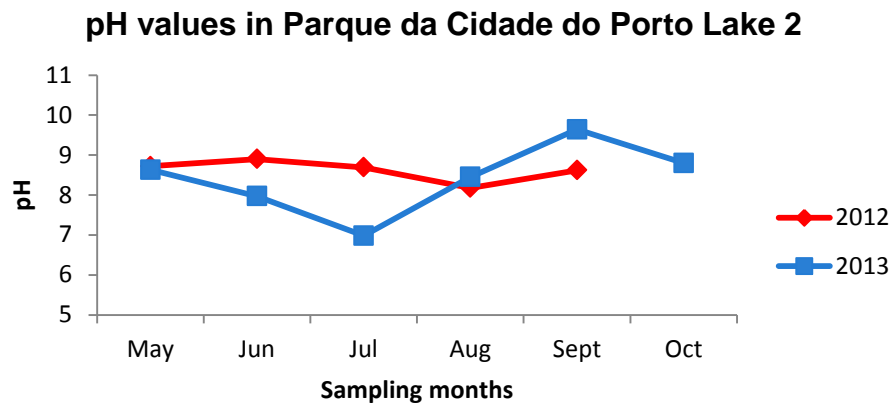


Figure 17. pH values registered in Parque da Cidade do Porto Lake 2 in the sampling periods of 2012 and 2013.

Parque da Cidade do Porto Lake 3

In figure 18, it is possible to observe an opposite variation in the recorded pH values of the 2 sampling periods. In 2012, pH values show a gradual and subtle tendency for the acidification of the environment; in 2013, there is a decrease of the pH values in June and July, comparing to May, followed by an increase of the same in August and September to stabilize in October.

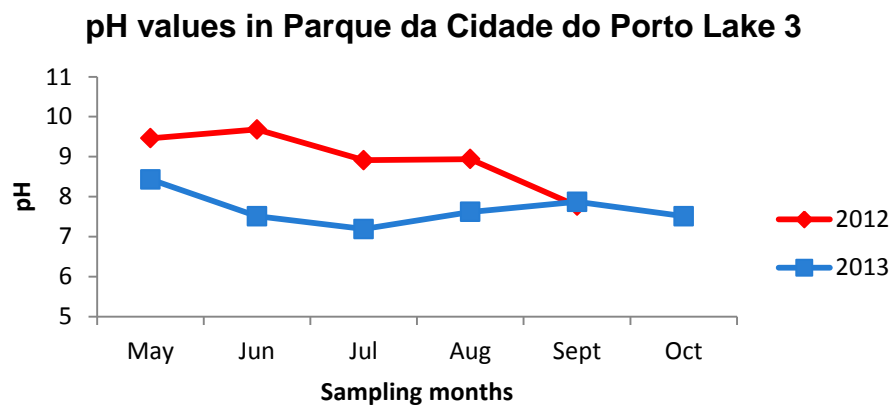


Figure 18. pH values registered in Parque da Cidade do Porto Lake 3 in the sampling periods of 2012 and 2013.

Vela Lake

In this sampling point, when compared the 2 sampling periods, it is possible to observe that there was a subtle seasonal variation in the pH values registered (Figure 19). Despite an opposite tendency recorded in August and September, the pH of the

water environment increases to time and slightly decrease in the late summer/autumn months (September and October).

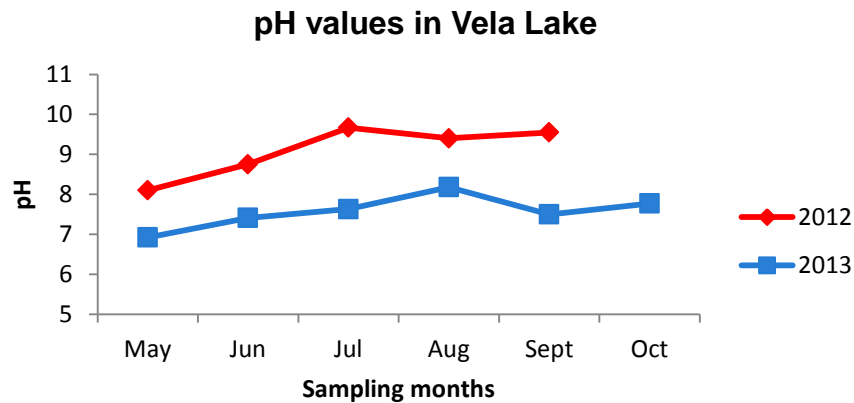


Figure 19. pH values registered in Vela Lake in the sampling periods of 2012 and 2013.

Mira Lake

In Mira Lake (Figure 20), there is not a consistent seasonal variability between sampling periods. On the contrary, it is registered an opposite tendency when compared the values recorded in each year. In 2012, was registered a slight increase in the values of May and June, followed by a posterior decrease of the values in the following months; in 2013, it was firstly recorded a decrease in pH values, followed by an increase in July and August and then, a new decrease of the values in September and October.

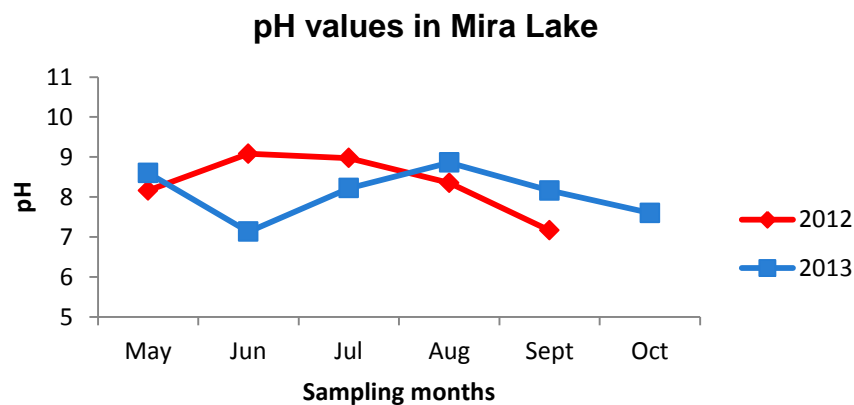


Figure 20. pH values registered in Mira Lake in the sampling periods of 2012 and 2013.

3.2. PCR amplification

After DNA extraction, the presence of genomic DNA was confirmed by agarose gel electrophoresis. Posteriorly, it was performed an analysis of 16S rRNA gene, to confirm the presence of cyanobacterial DNA in the extracted samples. After confirmation of the presence of the gene, amplification of the fragments involved in cyanotoxin biosynthesis was performed. PCR amplification was performed for all the samples, being the results summarized on Tables 2-15. The results for *mcyG*, *anaC-anab*, *pks* M4/M5, *ps* and AMT primer sets are not described, since they were negative for all tested samples.

Hepatotoxins

The presence of amplified fragments implicated in microcystin biosynthesis in every samples was detected. However, this detection was more frequent in the sampling period of 2012 than in the sampling period of 2013, where detection of the amplified fragments of *mcy* gene was null especially in the first months of the sampling period (May and June). The exception goes for October of 2013, where the detection of amplified fragments of *mcy* gene was complete in a total of 4 freshwater systems (Tables 3-6) and for Mira Lake, where the detection of *mcy* gene clusters was higher in 2013 than in 2012 (Table 8). The clusters *mcyA* and *mcyD* were the most detected in both sampling years. Detection of amplified fragments involved in cylindrospermopsin biosynthesis was only positive for *pks* (K18/M4) gene in a sample of Vela Lake from September 2012, being negative for the remaining samples.

Table 2. Detection of amplified fragments implicated in hepatotoxins biosynthesis from samples of Torrão Reservoir.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	-	-	-	-
	2013	-	-	-	-	-	-
Jun	2012	-	+	-	-	+	-
	2013	-	-	-	-	-	-
Jul	2012	-	+	-	-	+	-
	2013	-	+	-	+	-	-
Aug	2012	-	n.d.	n.d.	n.d.	n.d.	n.d.
	2013	-	-	-	(+)	(+)	-
Sept	2012	-	n.d.	n.d.	n.d.	n.d.	n.d.
	2013	-	+	+	+	+	+
Oct	2013	-	-	-	-	-	-

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 3. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Parque Fluvial do Tâmega.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	-	-	-	(+)
	2013	-	-	-	-	-	-
Jun	2012	-	+	(+)	+	+	-
	2013	-	-	-	-	-	-
Jul	2012	-	+	-	-	+	-
	2013	-	-	-	-	-	-
Aug	2012	-	+	-	-	(+)	-
	2013	-	-	-	(+)	-	(+)
Sept	2012	-	-	+	+	+	(+)
	2013	-	+	+	+	+	+
Oct	2013	-	+	+	+	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 4. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Parque da Cidade do Porto Lake 1.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	+	+	-	+	-
	2013	-	+	(+)	+	-	-
Jun	2012	-	+	+	-	+	+
	2013	-	+	+	+	+	+
Jul	2012	-	+	+	-	+	+
	2013	-	+	+	+	+	+
Aug	2012	-	+	+	+	+	+
	2013	-	+	+	+	+	+
Sept	2012	-	+	+	+	+	+
	2013	-	+	+	+	+	+
Oct	2013	-	+	+	+	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 5. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Parque da Cidade do Porto Lake 2.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	-	-	-	-
	2013	-	-	-	-	-	-
Jun	2012	-	-	-	-	-	-
	2013	-	-	-	-	-	-
Jul	2012	-	+	-	+	+	-
	2013	-	+	(+)	+	-	+
Aug	2012	-	-	-	-	+	-
	2013	-	+	+	+	+	+
Sept	2012	-	+	(+)	-	(+)	-
	2013	-	+	+	+	+	+
Oct	2013	-	+	+	+	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 6. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Parque da Cidade do Porto Lake 3.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	-	-	-	-
	2013	-	-	-	-	-	(+)
Jun	2012	-	+	+	-	+	-
	2013	-	-	-	-	-	-
Jul	2012	-	+	+	-	+	-
	2013	-	-	-	-	-	-
Aug	2012	-	(+)	-	-	(+)	-
	2013	-	-	-	-	-	-
Sept	2012	-	+	+	-	+	(+)
	2013	-	+	+	(+)	+	-
Oct	2013	-	+	+	+	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 7. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Vela Lake.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	+	(+)	+	(+)
	2013	-	-	-	-	-	+
Jun	2012	-	+	+	-	+	+
	2013	-	-	-	-	-	-
Jul	2012	-	+	+	+	+	+
	2013	-	+	-	+	-	-
Aug	2012	-	+	+	+	+	(+)
	2013	-	-	-	-	(+)	-
Sept	2012	+	+	+	-	+	+
	2013	-	-	-	-	-	-
Oct	2013	-	-	-	(+)	-	-

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 8. Detection of amplified fragments implicated in hepatatoxins biosynthesis from samples of Mira Lake.

<i>Month</i>	<i>Year</i>	<i>pks</i>	<i>mcyA</i>	<i>mcyB</i>	<i>mcyC</i>	<i>mcyD</i>	<i>mcyE</i>
May	2012	-	-	-	-	-	(+)
	2013	-	-	-	+	-	-
Jun	2012	-	-	-	-	-	-
	2013	-	-	-	+	-	+
Jul	2012	-	-	-	-	-	-
	2013	-	+	-	+	-	(+)
Aug	2012	-	+	-	-	-	-
	2013	-	-	-	+	(+)	+
Sept	2012	-	-	-	-	+	-
	2013	-	-	-	(+)	-	-
Oct	2013	-	-	-	(+)	-	(+)

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Neurotoxins

Amplified fragments implicated in neurotoxin biosynthesis were detected in most of tested samples. Regarding anatoxin-a analysis, amplification of the fragments occurred mostly in the samples from 2013 sampling period. Saxitoxin fragments were not so much present in the samples as anatoxin-a fragments but, like anatoxin-a, their

presence was registered mostly in the 2013 sampling period, especially in its final months (September and October).

Table 9. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Torrão Reservoir.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	+
	2013	-	+
June	2012	-	-
	2013	-	+
July	2012	-	-
	2013	+	+
August	2012	n.d.	n.d.
	2013	-	-
September	2012	n.d.	n.d.
	2013	(+)	+
October	2013	-	-

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 10. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Parque Fluvial do Tâmega.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	+
June	2012	-	-
	2013	-	+
July	2012	-	+
	2013	-	+
August	2012	-	+
	2013	-	-
September	2012	-	-
	2013	-	-
October	2013	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 11. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Parque da Cidade do Porto Lake 1.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	+
June	2012	-	-
	2013	-	+
July	2012	-	-
	2013	-	+
August	2012	-	-
	2013	-	-
September	2012	-	-
	2013	+	+
October	2013	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 12. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Parque da Cidade do Porto Lake 2.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	+
June	2012	+	-
	2013	+	+
July	2012	+	-
	2013	-	+
August	2012	+	-
	2013	+	-
September	2012	-	-
	2013	+	+
October	2013	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 13. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Parque da Cidade do Porto Lake 3.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	-
June	2012	+	-
	2013	-	+
July	2012	-	-
	2013	+	+
August	2012	-	-
	2013	+	+
September	2012	-	-
	2013	-	-
October	2013	+	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 14. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Vela Lake.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	-
June	2012	+	-
	2013	-	+
July	2012	+	(+)
	2013	-	+
August	2012	+	-
	2013	-	+
September	2012	+	-
	2013	-	-
October	2013	+	-

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

Table 15. Detection of amplified fragments implicated in neurotoxin biosynthesis from samples of Mira Lake.

<i>Month</i>	<i>Year</i>	<i>sxtI</i>	<i>anaC-gen</i>
May	2012	-	-
	2013	-	+
June	2012	-	-
	2013	-	+
July	2012	-	-
	2013	-	+
August	2012	+	-
	2013	+	-
September	2012	-	-
	2013	-	+
October	2013	-	+

[+] – Presence; [(+)] – Presence (fragment with low intensity); [-] – Absence; n.d. – no data

3.3. ELISA quantification assays

Cyanotoxin quantification by ELISA was performed for microcystin, cylindrospermopsin, anatoxin-a and saxitoxin. The immunoassay was performed for all samples that had amplified at least one gene involved in each cyanotoxin biosynthesis, except for cylindrospermopsin quantification of samples from Vela Lake, where all the samples were analyzed. The results are expressed in µg/L.

Microcystin

Quantification of microcystins was performed through a commercial kit (Abraxis, PA, USA), whose detection limits are between 0.15 µg/L and 5 µg/L. Samples that have quantified microcystin are summarized in the figures below (Figures 21-27). Results below the detection limit were considered as negative. Samples identified with (*) are diluted in a proportion of 1:10. Microcystin was quantified in all sampling points.

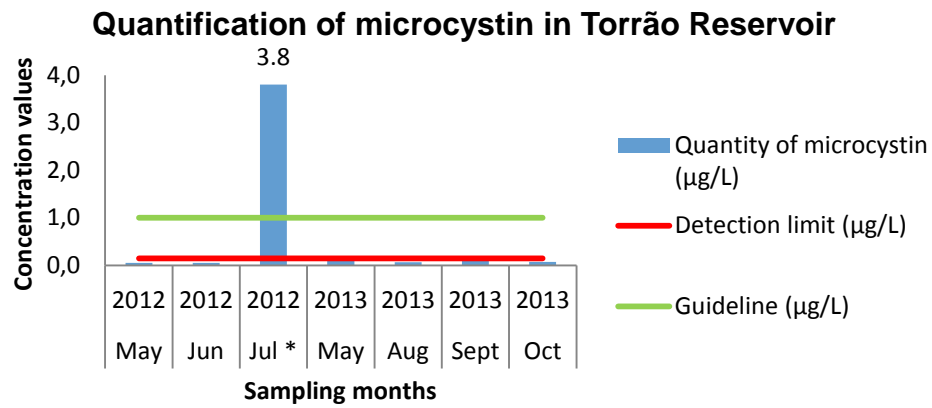


Figure 21. Microcystin quantification by ELISA in Torrão Reservoir in 2012 and 2013.

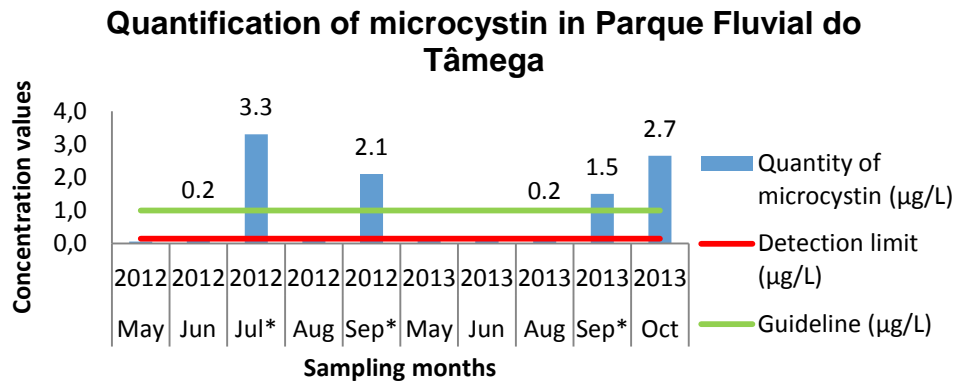


Figure 22. Microcystin quantification by ELISA in Parque Fluvial do Tâmega in 2012 and 2013.

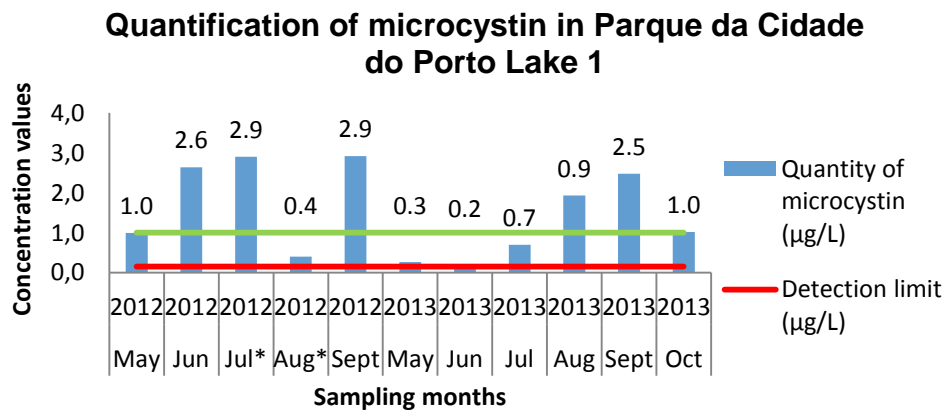


Figure 23. Microcystin quantification by ELISA in Parque da Cidade do Porto Lake 1 in 2012 and 2013.

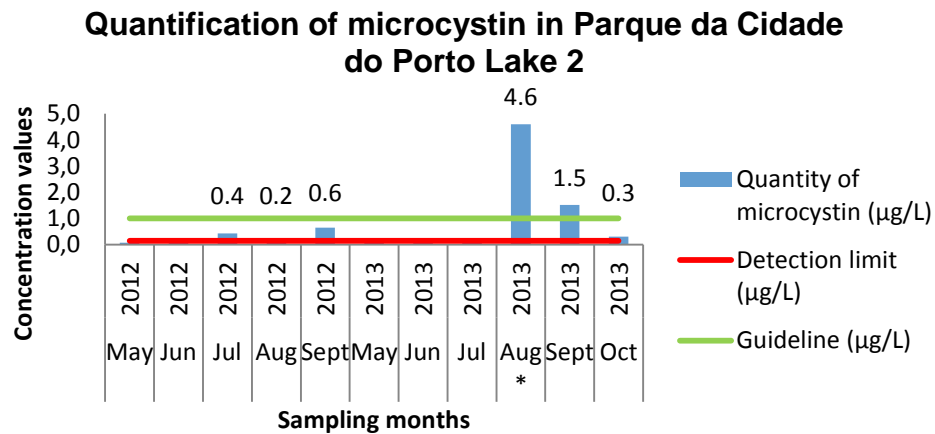


Figure 24. Microcystin quantification by ELISA in Parque da Cidade do Porto Lake 2 in 2012 and 2013.

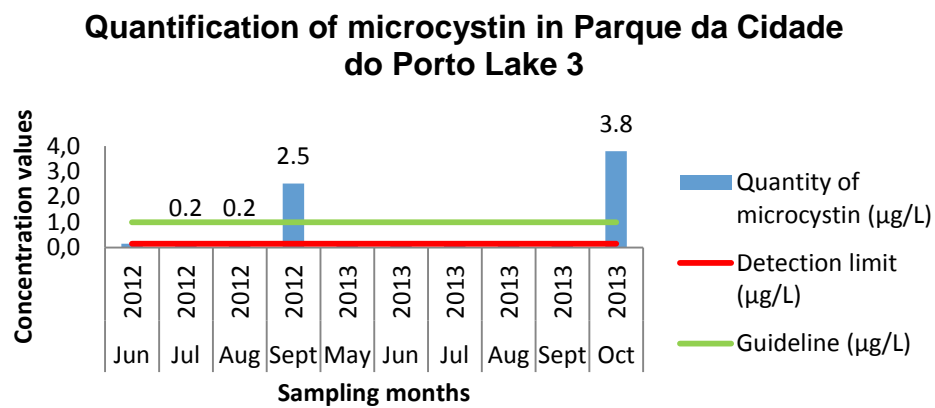


Figure 25. Microcystin quantification by ELISA in Parque da Cidade do Porto Lake 3 in 2012 and 2013

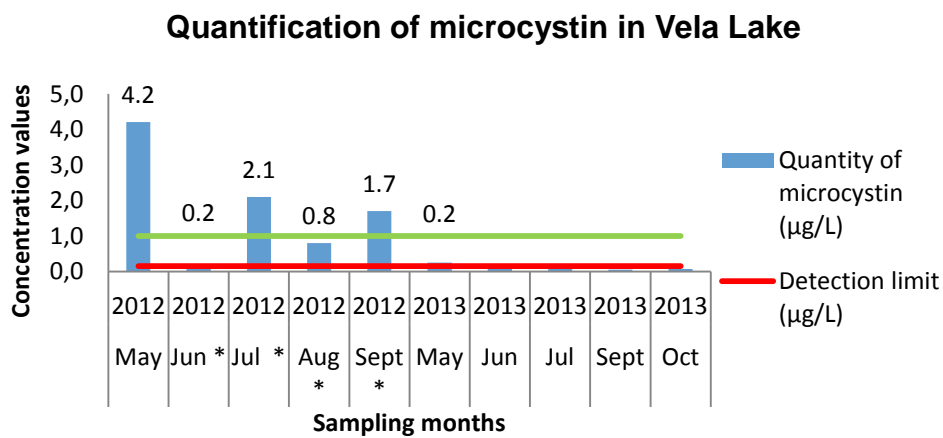


Figure 26. Microcystin quantification by ELISA in Vela Lake in 2012 and 2013.

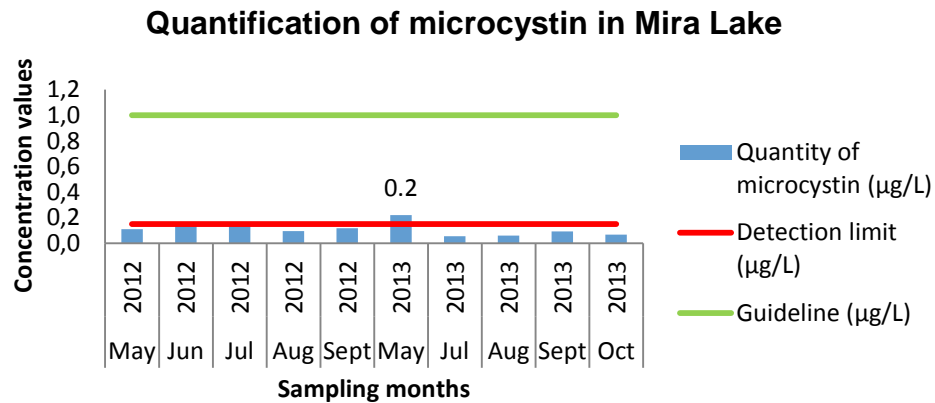


Figure 27. Microcystin quantification by ELISA in Mira Lake in 2012 and 2013.

Cylindrospermopsin

Cylindrospermopsin quantification in Vela Lake samples was performed through a commercial kit (Abraxis, PA, USA), with a range of detection between 0.05 µg/L and 2 µg/L. Samples that quantified cylindrospermopsin are summarized in Figure 28. Results below 0.05 µg/L were considered as negative. Two samples from August and September of 2012 quantified cylindrospermopsin with quantities of 0.3 and 0.6 µg/L.

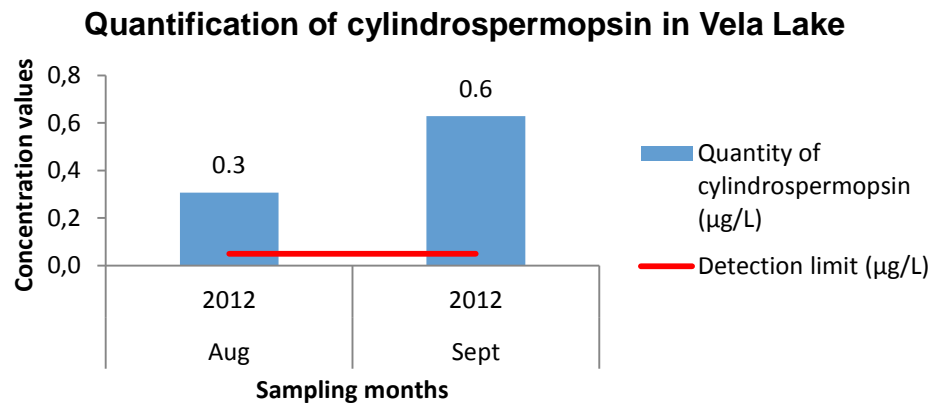


Figure 28. Cylindrospermopsin quantification by ELISA in Vela Lake in 2012.

Anatoxin-a

Quantification of anatoxin-a was only performed in samples that amplified a fragment implicated in anatoxin-a biosynthesis. For that, a commercial kit (Abraxis, PA, USA), with a range of detection between 10 µg/L and 500 µg/L was used. The results

are summarized in Figures 29-33. Sample with quantification values below the detection limit were considered as negative. The presence of anatoxin-a in quantifiable values is registered in the 2013 sampling period in Torrão Reservoir, Parque Fluvial do Tâmega, Lakes 1 and 3 of Parque da Cidade do Porto and Mira Lake.

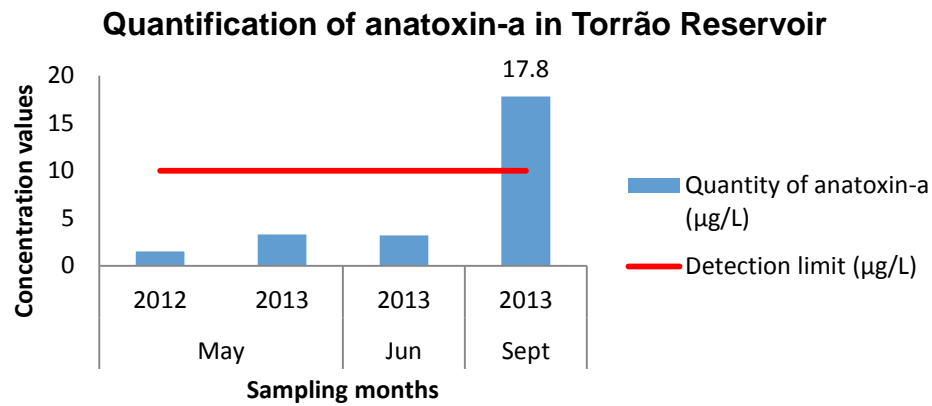


Figure 29. Anatoxin-a quantification by ELISA in Torrão Reservoir in 2012 and 2013.

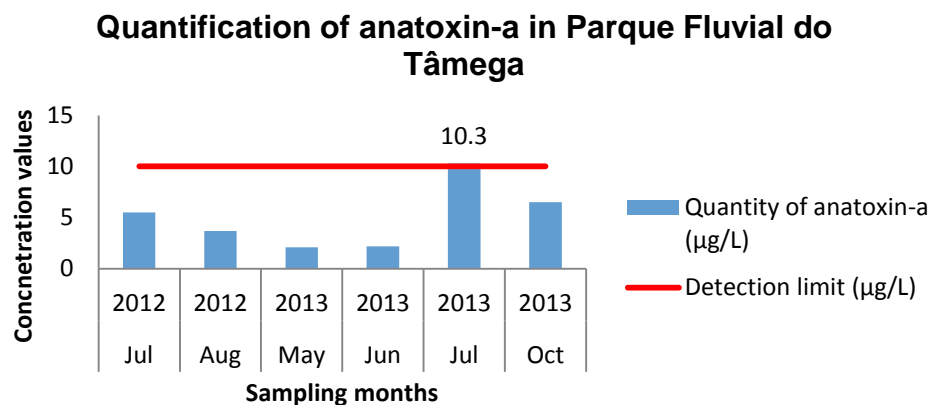


Figure 30. Anatoxin-a quantification by ELISA in Parque Fluvial do Tâmega in 2012 and 2013.

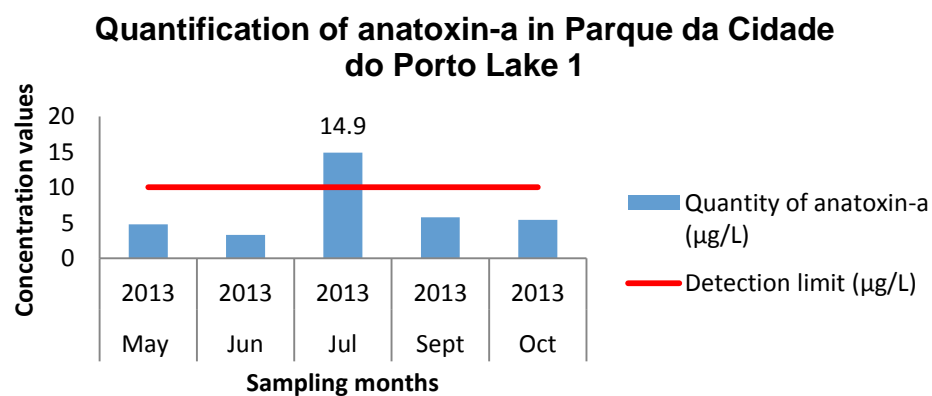


Figure 31. Anatoxin-a quantification by ELISA in Parque da Cidade do Porto Lake 1 in 2013.

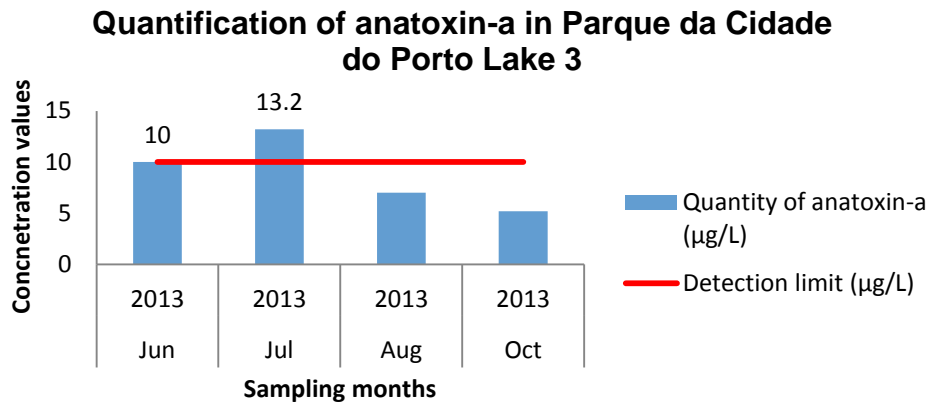


Figure 32. Anatoxin-a quantification by ELISA in Parque da Cidade do Porto Lake 3 in 2013.

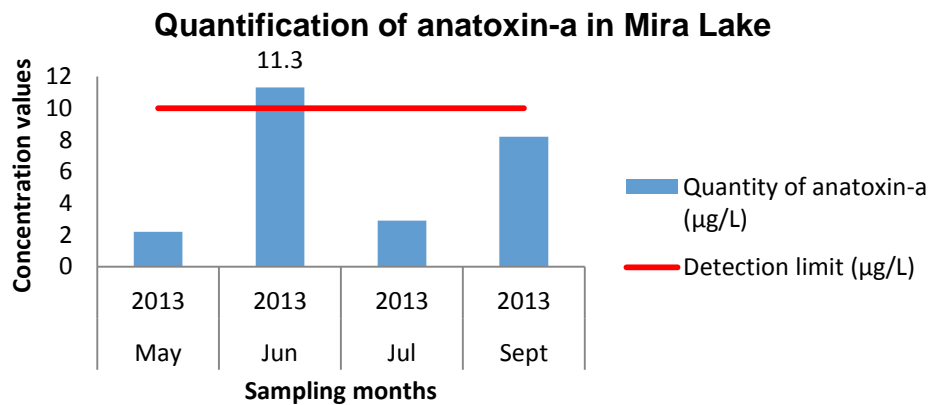


Figure 33. Anatoxin-a quantification by ELISA in Mira Lake in 2013.

Saxitoxin

Saxitoxin quantification was only performed in samples that amplified a fragment implicated in saxitoxin synthesis through the use of a commercial kit (Abraxis, PA, USA). The kit quantifies saxitoxin with values between 0.02 and 0.4 µg/L, being results below the detection limit considered as negative. Saxitoxin was only quantifiable in 2012 samples from Parque da Cidade do Porto Lake 2 and Vela Lake (Figures 34 and 35). Samples identified with an (*) are diluted in a proportion of 1:5, and samples identified with (**) are diluted in a proportion of 1:10.

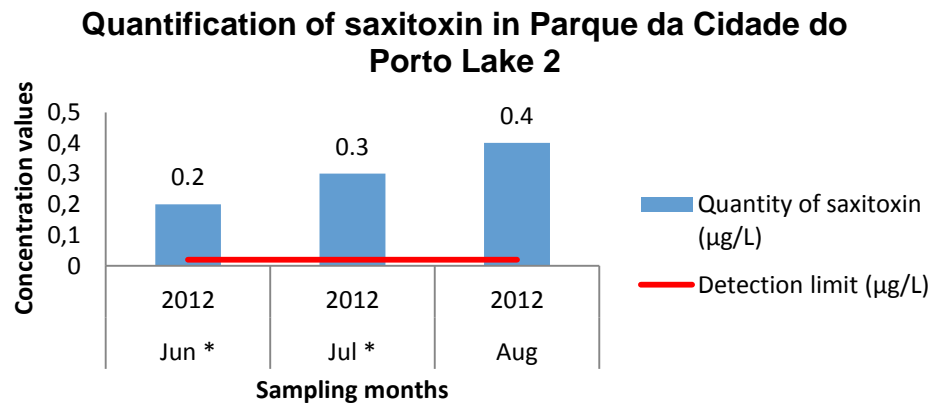


Figure 34. Saxitoxin quantification by ELISA in Parque da Cidade do Porto Lake 2 in 2012.

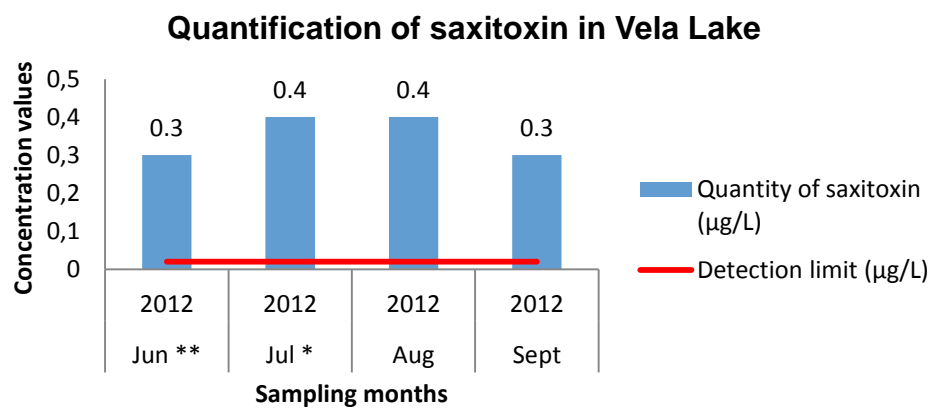


Figure 35. Saxitoxin quantification by ELISA in Vela Lake in 2012.

3.4. Detection and quantification of cylindrospermopsin by HPLC-PDA

The presence of cylindrospermopsin was detected by HPLC in samples from May, June and September 2012. Based on the analysis of the obtained chromatograms (Figures 36 and 37) it was possible to determine that the retention time was between 6.5-7.2 minutes in samples from 2012 and 8.2-9.2 minutes in 2013. This difference in standard peaks for cylindrospermopsin is due to the columns used. Despite the detection of cylindrospermopsin, the state of the columns determines the retention time for this toxin. In samples collected in 2012, it is possible to see in Figures 36 and 37 that the area of the peak correspond in the spectrum (Figures 36b) and 37b)) to cylindrospermopsin that is detected at 262 nm. In samples collected in 2013, it is

possible to observe (Figures 38 and 39) that the sample peaks do not correspond to standard peaks for cylindrospermopsin at 262 nm, thus confirming the absence of cylindrospermopsin for these samples. Quantification values of cylindrospermopsin that were obtained from 2012 samples are described below in Figure 40.

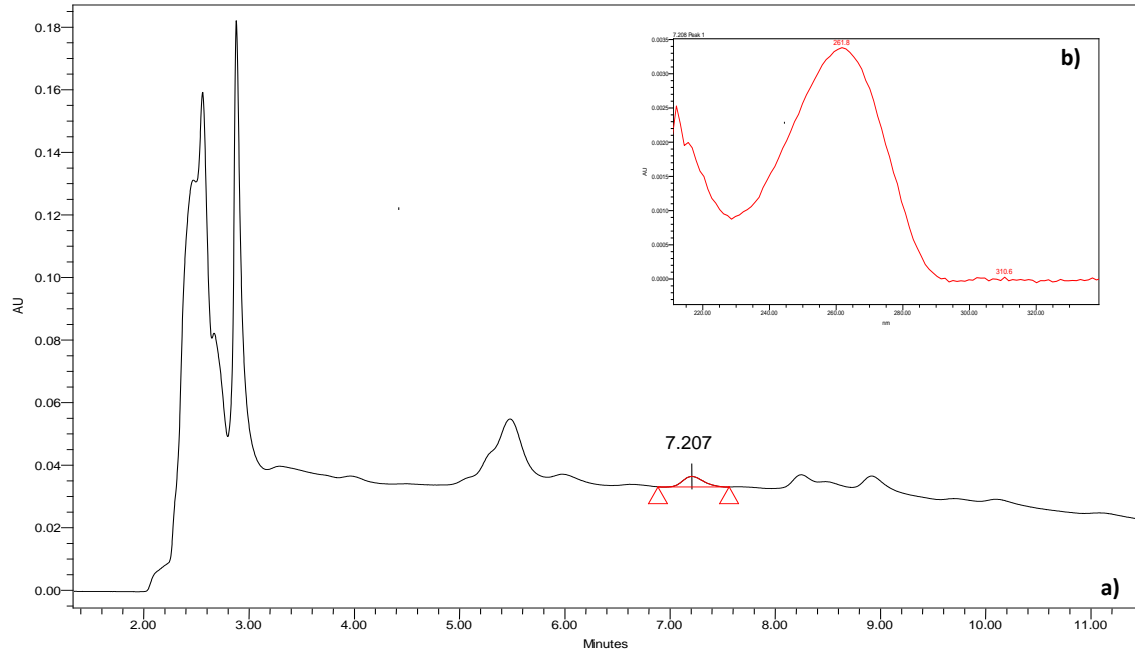


Figure 36. (a) Chromatogram of the sample from May 2012 of dissolved matter at 262 nm, with the retention time at 7.207 minutes. **(b)** Spectrum of absorption for cylindrospermopsin.

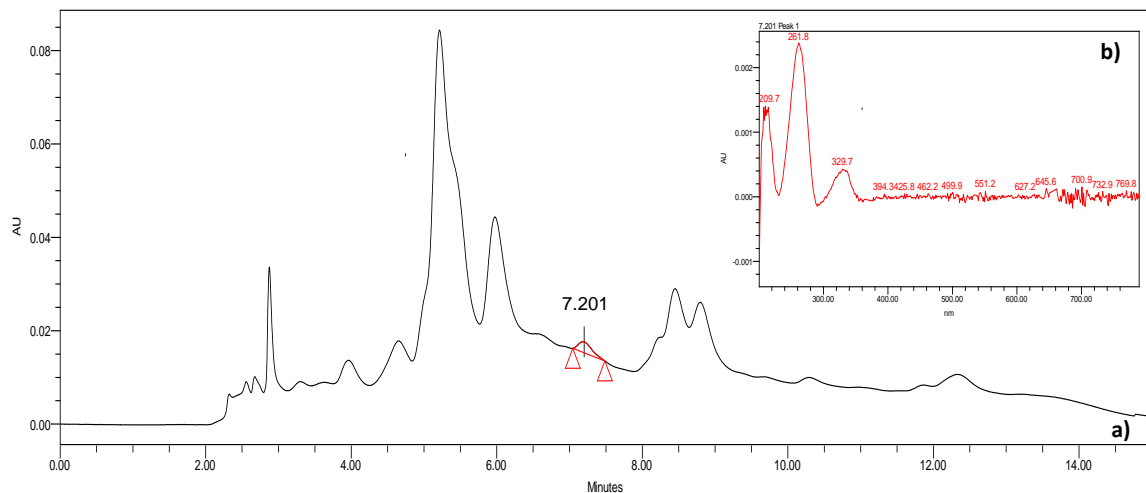


Figure 37. (a) Chromatogram of the sample from September 2012 for dissolved matter at 262 nm, with the retention time at 7.201 minutes. **(b)** Spectrum of absorption for cylindrospermopsin.

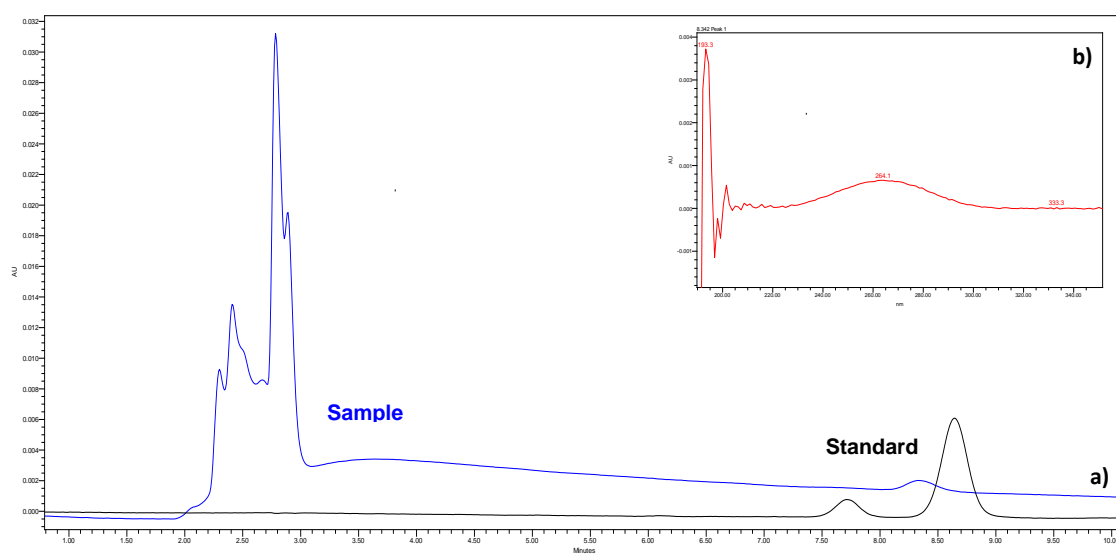


Figure 38. (a) Chromatogram of the sample from May 2013 of dissolved matter at 262 nm. **(b)** Spectrum of absorption for cylindrospermopsin.

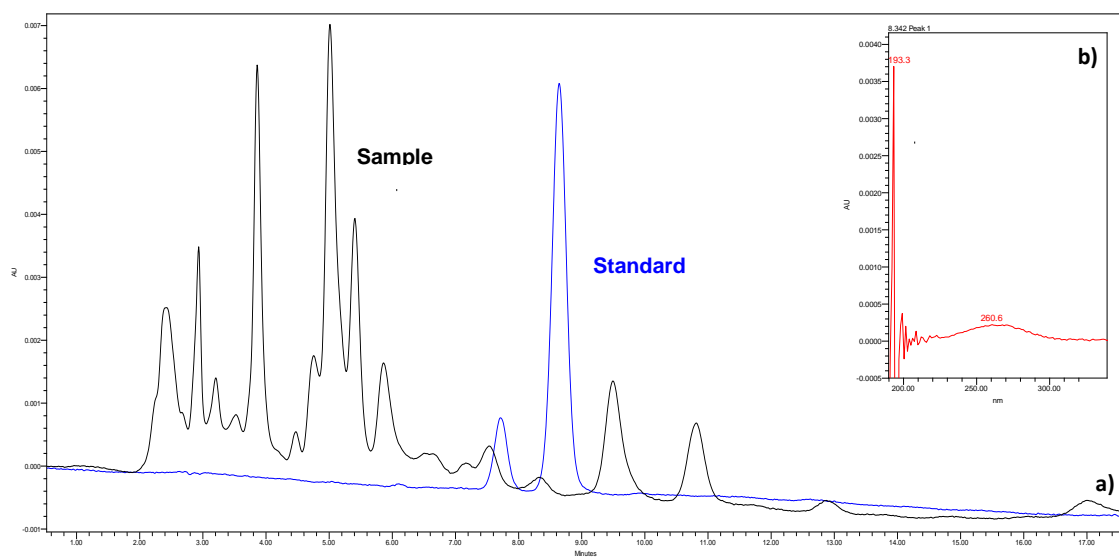


Figure 39. (a) Chromatogram of the sample from October 2013 for particulated matter at 262 nm. **(b)** Spectrum of absorption for cylindrospermopsin.

Cylindrospermopsin quantification by HPLC-PDA

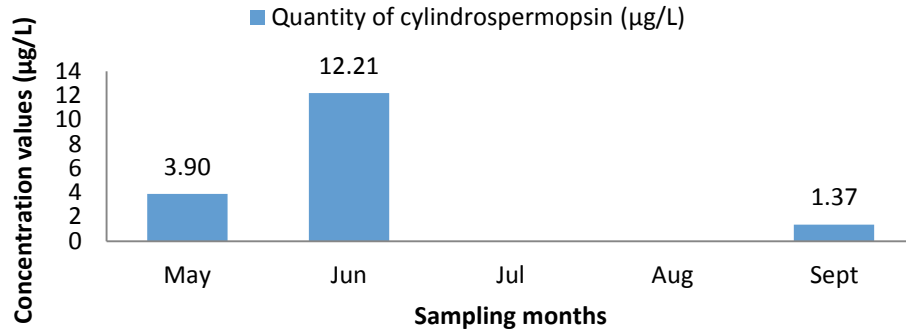


Figure 40 . Quantification values of cylindrospermopsin present in samples from Vela Lake obtained by HPLC-PDA.

3.5. Quantification by LC-MS

Analysis by LC-MS confirms the presence of cylindrospermopsin in Vela Lake samples from May, June and September of 2012 and the absence of the cyanotoxin in the samples from July and August of 2012 and samples from 2013. The spiked of the sample 22 (Figure 42) showed that the signal is not suppressed by the environmental matrix. The presence of cylindrospermopsin in samples was validated by performing the MS/MS method in the precursor ion (m/z 415.6) and by the detection of at least two of its fragment ions with m/z 336, 274, 194 and 175. The figures below are representative of the results that show the presence of cylindrospermopsin in 2012 samples (Figure 41) and the absence of the same toxin in 2013 samples (Figure 43).

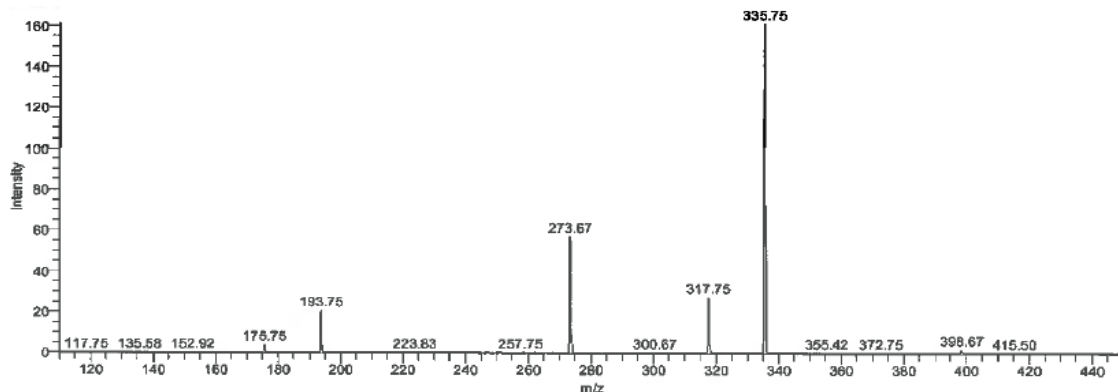


Figure 41. Mass spectrum of cylindrospermopsin by LC-MS (sample from May 2012).

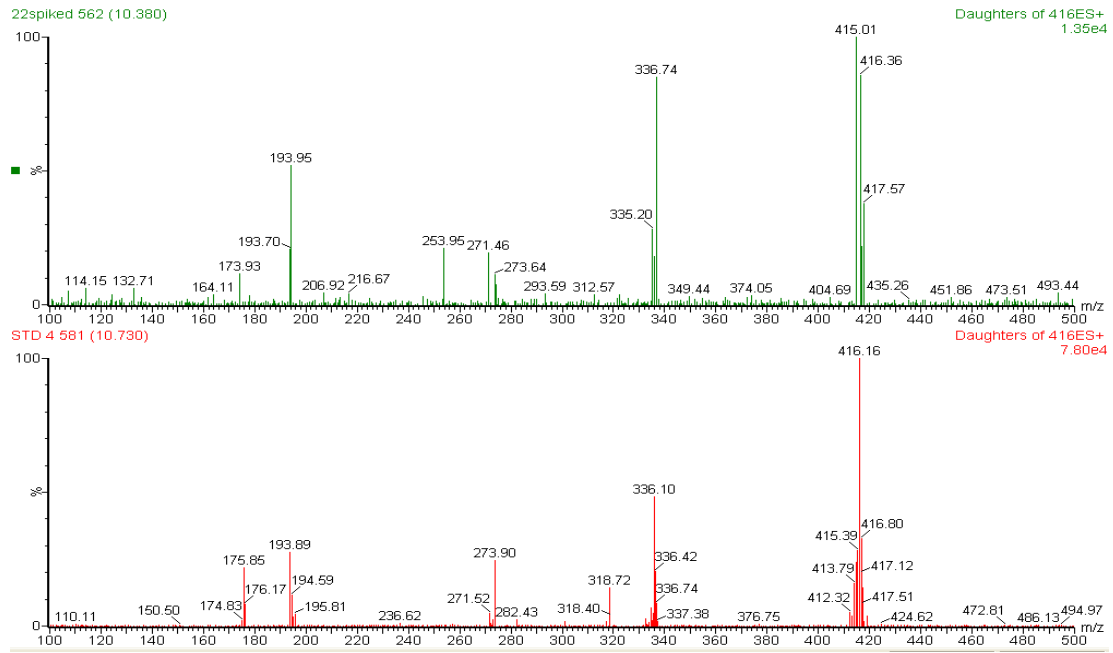


Figure 42. Mass spectra of cylindrospermopsin by LC-MS (spiked sample from October 2013 and standard).

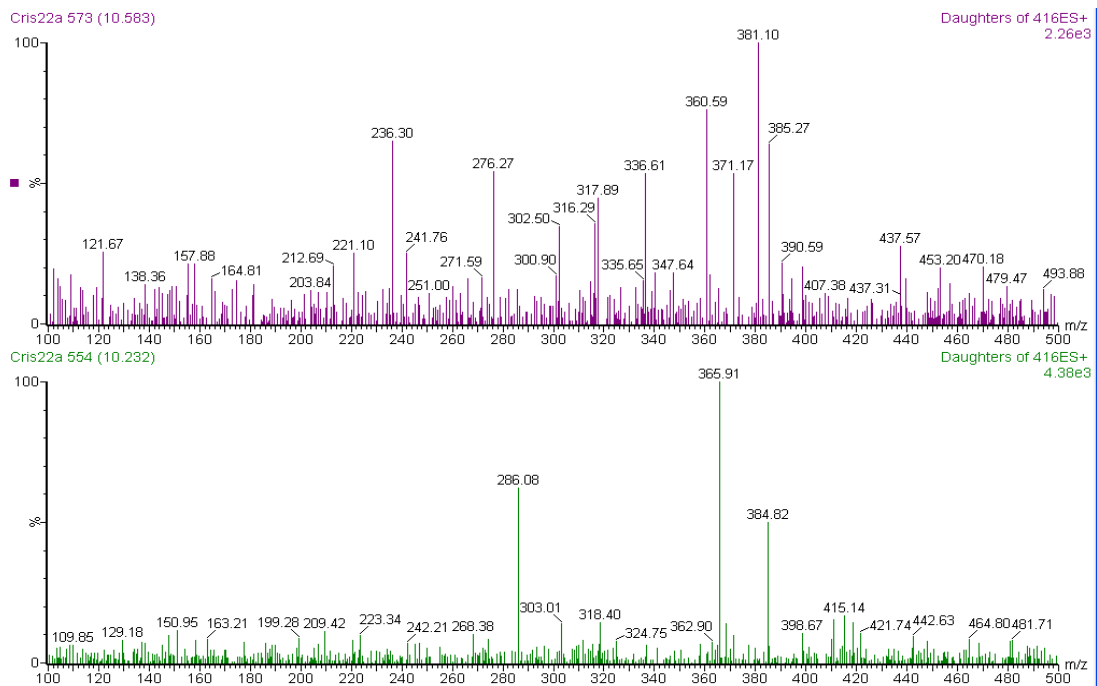


Figure 43. Mass spectra of cylindrospermopsin by LC-MS (samples from May 2013 and October 2013).

4. Discussion

The presence of cyanotoxins and/or their potentially-producing genera in several sampling sites shows that temperature and pH conditions were, at least, adequate for proliferation of cyanobacteria and for the production of their secondary metabolites, cyanotoxins. Variations in the physical/chemical parameters of the water environment originate large variation in the cyanobacteria community and in the quantities of cyanotoxins produced (Vasconcelos, 1993; Funari and Testal, 2008). According to the literature, the optimal conditions of temperature and pH for the development of the cyanobacteria communities are between 15°C and 30°C, and pH 6 and 9 (Vale, 2005).

Despite this study have used data of the atmospheric temperature, it is proven that atmospheric temperature influences water temperature (Vale, 2005), wherein higher atmospheric temperatures correspond to higher values in the water temperature. Maximum atmospheric temperatures recorded in the two years of sampling are within the range described above for the ideal conditions of development of cyanobacterial communities and cyanotoxin production; on the contrary, the registered minimum atmospheric temperatures are, sometimes, several degrees lower than the ideal period cited before. Comparing the temperatures recorded in 2012 and 2013, it is possible to conclude that in 2013 were observed higher maximum temperatures and lower minimum temperatures than in 2012, making the recorded temperature range higher in 2013 than in 2012. According to the literature above, and despite the lack of statistically significant differences between the analyzed data, this larger temperature range can cause several variations in cyanobacterial communities and, subsequently, the variation in the quantity of cyanotoxins produced.

Variations in the pH of the water are related to the nature of the water, soil and its surrounding area in terms of vegetation (Vale, 2005). Considerable differences are recorded when comparing the values recorded in each of the sampling periods. The pH values registered in 2012 allowed the classification of the water from the studied freshwater systems as alkaline. However, in 2013, the status of the water varied between slightly acidic and alkaline.

Higher pH values (between 7.5 to 9) are common during periods of formation of blooms of cyanobacteria (Regueiras, 2009), since cyanobacteria thrive best in environments with high pH (pH > 8.5), being frequent their appearance in eutrophic

systems, characteristic for their alkalinity, especially in the summer months (Oliva-Teles *et al.*, 2008; Summerfield and Sherman, 2008).

According to the literature, and by observation of the obtained results, it is possible to establish a relationship between the variation of pH in the aquatic environment and the presence of toxins. Unlike other studied cyanotoxins, anatoxin-a is easily degraded in highly alkaline water environments (Osswald *et al.*, 2007; Yavasoglu *et al.*, 2008), rather being present in neutral or slightly alkaline water systems. Therefore, anatoxin-a detection in quantifiable values occurred mainly in the 2013 sampling period, given the lower pH values registered in this sampling period. The decrease in the pH values can also explain the absence of cylindrospermopsin in water samples from Lake Vela collected in 2013 and the decrease in the presence of microcystin in samples taken in the same year in the several sampling sites, as they are usually cyanotoxins present in alkaline environments, given their chemical and molecular characteristics.

The application of molecular methods in the detection of cyanotoxin potentially-producer genera allows the analysis of water samples, without the cultivation of samples in laboratory. PCR technique is based on the amplification of fragments of the peptide chain involved in the synthesis of cyanotoxins, being a widely used method for the early detection of the potential for cyanotoxin production (Pearson and Neilan, 2008).

After confirmation of the presence of cyanobacterial DNA through the amplification of the 16S rRNA gene, amplification of the genes involved in the biosynthesis of microcystin (*mcy*) cylindrospermopsin (*pks*, *ps* and *AMT*), anatoxin-a (*anaC*) and saxitoxin (*sxt*) was performed, in order to determine the toxic potential of the genera present in the several freshwater systems under study.

Responsible for the toxicity of the microcystins produced, *mcy* gene clusters codify their synthesis chains (Bittencourt-Oliveira *et al.*, 2010; Kumar *et al.*, 2011). NRPS codifying genes, like *mcyA* and *mcyB* have been the most used clusters for the detection of hepatotoxic genotypes and NRPS/PKS *mcyE* gene cluster has been the most widely used cluster in the detection of microcystin potentially-producing genera, once it is essential for the synthesis of ADDA chain and for the activation and addition of D-glutamate to microcystin molecule (Ouahid, 2008), being this process essential for the level of toxicity of microcystin (Pearson and Neilan, 2008).

In this study, the presence of *mcy* gene cluster was registered in all sampling sites in both years. In the initial months (May and June) of each sampling period, *mcy* gene

amplification was negative for almost every sampling site, except for Lake 1 of Parque da Cidade do Porto, where *mcy* gene clusters were detected along both sampling periods. Comparing the results obtained by PCR with the results obtained with the ELISA tests, it is possible to conclude that *mcyA*, *mcyB* and *mcyD* were the most detected clusters in the samples tested for the presence of *mcy* gene clusters. The amplification of the *mcyB* gene cluster in most of the analyzed samples shows that there is a dominance of *Microcystis* spp. in the water, when regarding the presence of microcystin potentially-producing genera in the freshwater system. Despite the role of *mcyE* cluster in the toxicity of microcystin and in the synthesis of ADDA chain (Ouahid, 2008), it is possible to observe in this study that *mcyE* is not a limiting factor for microcystin production. It is also possible to conclude that the presence of, at least, one fragment of the *mcy* gene, does not exactly means that the production of microcystin occurs, but only the potential of the cyanobacteria present in the water for their production.

For this study, the detection of cylindrospermopsin-producing genera was performed through the amplification of three fragments from the cylindrospermopsin gene: Amidinotransferase (AMT), responsible for the formation of guanidinoacetate by transfer of the guanidine group; a PKS module, responsible for the elongation of the carbon chain, through the addition of acetate molecules; and a PS module (Kellmann *et al.*, 2006; Mihali *et al.*, 2008; Pearson and Neilan, 2010; Moreira *et al.*, 2011). A previous study (Schembri *et al.*, 2001), demonstrated the existence of a direct relationship between the production of cylindrospermopsin and the presence of NRPS and PKS peptides in *Cylindrospermopsis raciborskii*, thus raising the hypothesis that these two peptides are directly involved in the production of this cyanotoxin.

The obtained results showed that there was only amplification of the PKS gene cluster in the sample of September of 2012 from Vela Lake. However, the results from HPLC-PDA, LC-MS showed the presence of cyanotoxin in quantifiable quantities in samples from May, June and September of 2012 and results from ELISA tests showed the presence of the toxin in samples from August and September of 2012. The difference in the results, regarding the months where cylindrospermopsin was detected, could be explained by the detection limit of ELISA, that is 1000 higher than the detection limit of HPLC and by the higher amount of samples needed for cyanotoxin quantification by HPLC. Given the obtained results, it is possible that new primers with fragments that are involved in the cylindrospermopsin biosynthesis, for the detection of cylindrospermopsin-producing genera are needed in order to perform new analysis of these samples. Although it is also possible that, in the dates of the

collection, only the toxin was present in the water environment due to a previous senescence of the cells and the subsequent release of the cell content into the water.

Detection of anatoxin-a potentially-producing genera was performed through the amplification of *anaC* gene. According to the bibliography, *anaC* gene cluster is responsible for the initial step in anatoxin-a biosynthesis, proline adenylation, with the encoding of the AnaC protein (Rantala-Yilmén *et al.*, 2011). Its detection was registered in all samples sites in 2013 water samples, with exception for Torrão Reservoir, Parque Fluvial do Tâmega and Vela Lake that also had amplified fragments in samples of May, July and August of 2012. These results show the potential increase for the production of this toxin, later confirmed by ELISA in samples from June, July and September 2013.

Amplification of *sxt* gene cluster was recorded in all sampling sites in 2013, and in Lake 2 of Parque da Cidade do Porto, Vela Lake and Mira Lake in samples from June, July, August and September of 2012. Despite the register of amplified fragments in 2013, they only indicate the potential for saxitoxin production on those sampling sites, given that none of 2013 samples were quantified by ELISA. Comparing the two sampling periods, it can be observed that it is possible that abiotic factors like temperature and pH, along with other factors, lead to stop the cyanobacteria production of saxitoxin.

Chemical methods for detection and quantification of cylindrospermopsin have been widely used, due to its specificity and selectivity. In this study, application of HPLC-PDA and LC-MS techniques to detect and quantify cylindrospermopsin were only made in Vela Lake samples. A previous study (Moreira *et al.*, 2011) registered the presence of amplified fragments of a PKS gene in samples from this freshwater system and indicated the potential for the production of this cyanotoxin.

The presence of cylindrospermopsin was reported in three samples collected in May, June and September of 2012. In samples from May and September, the detection was observed in the results of the analysis of the dissolved matter; in samples from June, cylindrospermopsin was only detected in the particulate matter. Comparing with the obtained results from PCR amplification, it is possible to observe that, in September, was recorded the production of cylindrospermopsin from the cyanobacteria that were present in water; in samples from May and June, there is only the detection of the toxin, with no record of the presence of producing genera in the water. This raises the hypothesis of the occurrence of a cyanobacterial senescence process and

there were the release of the cellular content into the water, before the collection of the samples.

The use of biochemical methods, like ELISA, are characterized by the use of commercial kits composed by mono or polyclonal antibodies in order to quantify the concentration of cyanotoxins in water samples, by direct or indirect competition of cyanotoxin molecules with their specific antibodies for the binding receptors located in the plate (Rivasseau *et al.*, 1999; Rapala *et al.*, 2002; Mathys and Surholt, 2004; Sangolkar *et al.*, 2006). Its lower detection limit as well as their capacity to detect cyanotoxins within the legal limits, makes biochemical methods the primary methods for a rapid quantification of the concentration of cyanotoxins in water samples.

Based on the results of PCR amplification, quantification of cyanotoxins with ELISA were performed in samples that showed amplified fragments of the genes used for the detection of cyanotoxin potentially-producing genera.

For the quantification of microcystin, concentration values for MC-LR equivalent were calculated, given that this test does not differentiate the several microcystin isoforms. The results show the presence of microcystin in quantifiable values in all sampling sites. The incidence of microcystin is higher in 2012 than in 2013, going according with PCR results and the differences and temperature and pH registered above. The presence of microcystins in water samples was registered mainly in samples from the months of July, August and September, except for Mira, where there is only a report of microcystin quantification in the sample of May 2013; and except for Lake 1 of Parque da Cidade do Porto, where, in both sampling periods, all the samples collected quantified microcystin. Concentration values for microcystin are, in general, above the legal limit of 1 µg/L established for drinking water. This could represent a public health problem, given the fact that water from Torrão Reservoir is used for human consumption. Despite the legal limit for water used for recreational purposes is established in 10 µg/L, the concentration of microcystins in water could still represent a serious public health problem, due to the use of this freshwater systems for water activities and for recreational fishing.

Possessing a lower detection limit than HPLC, ELISA tests for quantification of cylindrospermopsin were performed in Vela Lake samples, in order to compare the results obtained by both methods. Cylindrospermopsin was quantified in samples from August and September of 2012. Comparing both results, there is a lack of concordance in these results. This can be explained by the differences in the detection limits, where ELISA has a lower LOD than HPLC, which allows the quantification of smaller

concentration values of the toxin; it can also be explained by the volume collected for each method. For ELISA tests, 15 mL were collected of water from Vela Lake in each sampling month; for analysis by HPLC and LC-MS it was collected 5000 mL of water in each sampling month. The higher volume of sample allows a higher concentration of toxin, unlike what occurs with smaller samples.

The concentration of anatoxin-a in the water samples was only quantifiable in samples from 2013 sampling period in all sampling sites, except for the Lake 2 of Parque da Cidade do Porto and Vela Lake, that only possess the potential for anatoxin-a production. With exception of the Torrão Reservoir, where it was quantifiable in September, there were only results in samples from June and July. These results in line with the bibliography, suggest that anatoxin-a is easily degraded in high temperature and pH (Osswald *et al.*, 2007; Yavasoglu *et al.*, 2008).

Saxitoxin quantification in Portuguese freshwater systems was only recorded in samples of 2012 from Lake 2 of Parque da Cidade do Porto and Vela Lake, despite the presence of potentially-producing genera in all sampling sites in both sampling periods. This can be related to the production of saxitoxin in environments with higher values of pH and lower temperature ranges than the ones registered in 2013.

It can be observed, by comparison of the results of the ELISA tests with the results from PCR amplification, that despite the positive result of the amplification of the fragment in PCR, it does not necessarily means that there is the production of the toxin, although the quantification of the cyanotoxin in study is normally associated with the presence of the gene clusters involved in the biosynthesis of the toxin.

Ultimately, this study seems to be the first report of cylindrospermopsin, anatoxin-a and saxitoxin in freshwater systems from Portugal. Thus, there is the need for a complete monitoring program for these cyanotoxins, encompassing molecular, chemical and biochemical methods. However, and given the results obtained, it is possible to use molecular methods as an initial step for the monitoring and prevention of cyanotoxin production and action in the environment and organisms.

5. Conclusion

Given the obtained data, this study provides the first report of the presence of cylindrospermopsin, anatoxin-a and saxitoxin in Portuguese freshwater systems. It is also possible to conclude that the concentration of cylindrospermopsin quantified by HPLC-PDA are above the guideline for drinking water proposed by Humpage and Falconer (2003) of 1 µg/L of cylindrospermopsin, thus reinforcing the need for continuous monitoring programs for this type of toxin. The presence of anatoxin-a in quantifiable values creates the necessity of establishment of guidelines for its presence in water, in similarity with what occurs in countries like New Zealand, where the established guideline for anatoxin-a in drinking water is 6.0 µg/L. Regarding the presence of saxitoxin in the studied freshwater systems, despite the values registered by ELISA are below the guidelines established in New Zealand, Australia and Brazil (3 µg/L), the presence of this cyanotoxin in Portuguese freshwater systems creates the necessity of continuous monitoring of this toxin, as well as the creation of regulation for its presence in water.

This study concludes that an approach that encompasses chemical and biochemical methods for the quantification of cyanotoxins is needed. Differences in the specificity and sensitivity of the several methods used allowed a more accurate and precise comparative study of the cyanotoxins in the system, especially cylindrospermopsin. The inclusion of molecular methods as a first step in the monitoring programs of cyanotoxins surveillance should be considered. The accuracy of this methods in the detection of potentially toxic cyanobacterial strains prove to be an effective tool for an initial study of the freshwater systems, which allows an early preventive action in the ecosystem in a more effective and accurate way.

Nevertheless, it must be emphasized the importance of creating complete monitoring programs, including the several methods used for the detection and quantification of cyanotoxins and their producing cyanobacteria genera, but considering the molecular methods as the initial selected screening method. It is also important to create legislation that regulates the maximum amount of cyanotoxins in drinking and recreational water, in similarity to what occurs in countries like Australia and New Zealand.

6.Future Perspectives

As future work, is suggested the performance of HPLC for the detection and quantification of neurotoxins (anatoxin-a and saxitoxin), for comparison with the quantification values obtained with the ELISA assays; it is also suggested the sequencing of the amplified fragments acquired by PCR in order to determine the producing genera of the cyanotoxin in study; and the cultivation of the cyanobacteria present in the water samples collected from the several studied sampling sites, with the purpose of conduction toxicity tests. Given the obtained results, it is recommended the extension of this study to other freshwater systems in Portugal.

There is also the need to establish monitoring programs for freshwater systems that aim in the detection of cyanotoxins in the water and their producing genera in order to prevent its occurrence and its effects on organisms and the environment.

7. Communications

Rita Mendes, Cristiana Moreira, Vitor Vasconcelos and Agostinho Antunes (2013). *Molecular Genetics in Cyanotoxin Research – An Overview*.
Poster presentation in XXXVIII Portuguese Genetics Conference. 4th to 5th of June 2013, Porto, Portugal.

Rita Mendes, Cristiana Moreira, Vitor Vasconcelos and Agostinho Antunes (2013). *Cyanotoxin detection in Portuguese freshwater systems*.
Poster presentation in ICTC9 – Living with toxigenic Cyanobacteria – 9th International Conference on Toxic Cyanobacteria. 11th to 16th of August 2013, Johannesburg, South Africa.

Rita Mendes, Joana Azevedo, Cristiana Moreira, Vitor Vasconcelos and Agostinho Antunes (2013). *First report of Cylindrospermopsin in a Portuguese freshwater system*.
Poster presentation in ICTC9 – Living with toxigenic Cyanobacteria – 9th International Conference on Toxic Cyanobacteria. 11th to 16th of August 2013, Johannesburg, South Africa.

References

- Abrantes N., S.C. Antunes, M.J. Pereira and F. Gonçalves (2006). Seasonal succession of cladocerans and phytoplankton and their interactions in a shallow eutrophic lake (Lake Vela, Portugal). *Acta Oecologica*, 29(1):54-64.
- Aráoz, R., J. Molgó and N.T. Marsac (2010). Neurotoxic cyanobacterial toxins. *Toxicon* 56(5): 813-828.
- Artigas, M.L., P.J.V. Vale, S.S. Gomes, M.J. Botelho, S.M.Rodrigues and A. Amorim (2007). Profiles of paralytic shellfish poisoning toxins in shellfish from Portugal explained by carbamoylase activity. *Journal of Chromatography A* 1160(1-2): 99-105.
- Baker J.A, B. Entsch, B.A Neilan and D.B.McKay (2002). Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. *Applied and Environmental Microbiology*, 68 (12):6070-6076.
- Ballot, A., J. Fastner, M. Lentz and C. Wiedner (2010). First report of anatoxin-a producing cyanobacterium *Aphanizomenon issatschenkoi* in northeastern Germany. *Toxicon* 56: 964-971.
- Bittencourt-Oliveira, M.C (2003). Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a *mcyB* molecular marker. *Harmful Algae* 2: 51-60.
- Bittencourt-Oliveira, M.C, D.M.S Santos and N.A Moura (2010a). Toxic cyanobacteria in reservoirs in northeastern Brazil: detection using a molecular method. *Brazilian Journal of Biology* 4: 1005-1010.
- Bittencourt-Oliveira, M.C., V. Piccin-Santos and S. Gouvêa-Barros (2010b). Microcystin-producing genotypes from cyanobacteria in Brazilian reservoirs. *Environmental Toxicology* 27: 461-471.
- Burch, M.D. (2008). Effective doses, guidelines & regulations. *Advances in Experimental Medicine and Biology* 619: 831-853
- Carmichael, W.W. and R. Liu (2006). Cyanobacteria toxins in the Salton Sea. *Saline Systems* 2: 1-13.
- Castro, B.B., S.C. Antunes, R. Pereira, A.M.V.M. Soares and F. Gonçalves (2005). Rotifer community structure in three shallow lakes: seasonal fluctuations and explanatory factors. *Hydrobiologia* 543: 221–232.

- Chen, J., J. Dai, H. Zhang, C. Wang, G. Zhou, Z. Han and Z. Liu (2010). Bioaccumulation of microcystin and its oxidative stress in the apple (*Malus pumila*). *Ecotoxicology* 19: 796-803.
- De Figueiredo, D.R., U.M. Azeiteiro, S.M. Esteves, F.J.M Gonçalves and M.J. Pereira (2004). Microcystin-producing blooms – a serious global public health issue. *Ecotoxicology and Environmental Safety* 59: 151-163.
- De Hoyos, C., A.I. Negro and J.J. Aldasoro (2004). Cyanobacteria distribution and abundance in the Spanish water reservoirs during thermal stratification. *Limnetica* 23: 119-132.
- Decreto-Lei nº 306/2007 de 27 de Agosto. *Diário da República*, 1.^a série - N.º 164. Ministério do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional.
- Ferreira, F.M B., J.M F. Soler, M.L. Fidalgo and P. Fernández-Vila (2001). PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). *Toxicon* 39: 757-761.
- Freitas, M. (2009). Monitorização de cianobactérias e cianotoxinas nas lagoas Portuguesas de Mira e Vela comparando métodos moleculares, imunológicos e volumes de amostragem. *Dissertação de Mestrado em Contaminação e Toxicologia Ambientais*. Universidade do Porto, Porto, Portugal, 118 pp.
- Funari E. and E. Testai (2008). Human health risk assessment related to cyanotoxins exposure. *Critical Reviews in Toxicology* 38(2):97 - 125.
- Gonçalves F., R. Ribeiro, V.M. Vasconcelos and A.M.V.M. Soares (1996). Anthropogenic influences on seasonal changes of nutrients, physical and chemical factors in three coastal freshwater shallow lakes (Portugal). *Limnetica*, 12 (2):47-52.
- Gonçalves, V., P. Raposeiro, J. Porteiro, M. Alves and M. Medeiros (2006). Proposta de definição de ecótipos e classificação preliminar do estado ecológico das lagoas dos Açores. Universidade dos Açores. Ponta Delgada.
- Gutiérrez-Praena, D., A. Jos, S. Pichardo, I.M. Moreno and A.M. Cameón (2013). Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A Review. *Food and Chemical Toxicology* 53: 139-152.
- Humpage, A.R., M. Fenech, P. Thomas and I.R. Falconer (2000). Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic aneugenic action of the cyanobacterial toxin, cylindrospermopsin.

- Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 472(1-2): 155-161.
- Humpage A.R. and I.R. Falconer (2003). Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline value. *Environmental Toxicology* 18(2):94-103.
- Kalaitzis, J.A., R. Chau, G.S. Kohli, S.A. Murray and B A. Neilan (2010). Biosynthesis of toxic naturally-occurring seafood contaminants. *Toxicon* 56(2): 244-258.
- Kellmann, R., T. Mills and B.A. Neilan (2006). Functional modeling and phylogenetic distribution of putative cylindrospermopsin biosynthesis enzymes. *Journal of Molecular Evolution* 62: 267-280.
- Kumar, A., A. Kumar, A.K. Rai and M.B. Tyagi (2011). PCR-based detection of *mcy* genes in blooms of *Microcystis* and extracellular DNA of pond water. *African Journal of Microbiology Research* 5(4): 374-381.
- Landsberg, J. (2002). The effects of harmful algal *blooms* on aquatic organisms. *Reviews in Fisheries Science* 10(2): 113-390.
- Llewellyn, L.E. (2006). Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Natural Product Reports* 23(2): 200-222.
- Lopes, V.R., V. Ramos, A. Martins, M. Sousa, M. Welker, A. Antunes and V. Vasconcelos (2012). Phylogenetic, chemical and morphological diversity of cyanobacteria from Portuguese temperate estuaries. *Marine Environmental Research* 73: 7-16.
- Mankiewicz-Boczek, J., K. Izydorczyk, Z. Romanowska-Duda, T. Jurczak, K. Stefaniak and M. Kokocinski (2006). Detection and monitoring toxigenicity of cyanobacteria by application of molecular methods. *Environmental Toxicology* 21: 380-387.
- Martins, R., P. Pereira, M. Welker, J. Fastner and V. Vasconcelos (2005). Toxicity of culturable cyanobacteria strains isolated from the Portuguese coast. *Toxicon* 46(4): 454-464.
- Mathys W. and B. Surholt (2004). Analysis of microcystins in freshwater samples using high performance liquid chromatography and an enzyme-linked immunosorbent assay. *International Journal of Hygiene and Environmental Health* 207(6):601-605.
- Mendes, B. and J.F.S. Oliveira; (2004). *Qualidade da água para consumo humano*. Lidel, Lisboa, 626pp.

- Merel, S., D. Walker, R. Chicana, S. Snyder, E. Baurès and O. Thomas (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environment International* 59: 303-327.
- Metcalf J.S. and G.A. Codd (2003). Analysis of cyanobacterial toxins by immunological methods. *Chemical Research in Toxicology* 16(2):103-112.
- Mihali, T.K., R. Kellmann, J. Muenchoff, K.D. Barrow and B.A. Neilan (2008). Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. *Applied and Environmental Microbiology* 74: 716-722.
- Morais, J. (2009). Avaliação do risco de ocorrência de cianobactérias tóxicas nos lagos do Parque da Cidade do Porto. *Dissertação de mestrado em Biologia e Gestão da Qualidade da Água*. Universidade do Porto, Porto, Portugal, 52 pp.
- Moreira, J., 1998. Depuração de Sistemas Aquáticos com Macrófitas. Universidade Técnica de Lisboa, Lisboa, Portugal.
- Moreira, C., A. Martins, J. Azevedo, M. Freitas, A. Regueiras, M. Vale, A. Antunes and V. Vasconcelos (2011). Application of real-time PCR in the assessment of the toxic cyanobacterium *Cylindrospermopsis raciborskii* abundance and toxicological potential. *Applied Microbiology and Biotechnology* 92: 189-197.
- Moreira, C., J. Azevedo, A. Antunes and V. Vasconcelos (2012). Cylindrospermopsin: occurrence, methods of detection and toxicology. *Journal of Applied Microbiology* 114: 605-620.
- Moreira, C., V. Ramos, J. Azevedo and V. Vasconcelos (2014). Methods to detect cyanobacteria and their toxins in the environment. *Applied Microbiology and Biotechnology* 98: 8073-8082.
- Msagati T.A.M, B.A. Siame and D.D. Shushu. 2006. Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. *Aquatic Toxicology* 78(4):382-397.
- Neilan, B.A., E. Dittmann, L. Rouhianen, R.A. Bass, V. Schaub, K. Sivonen and T. Börner (1999). Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. *Journal of Bacteriology* 181: 4089-4097.
- Neilan, B.A., L.A. Pearson, J. Muenchoff, M.C. Moffitt and E. Dittmann (2013). Environmental conditions that influence toxin biosynthesis in cyanobacteria. *Environmental Microbiology* 15(5): 1239-1253.
- Nimptsch, J., C. Wiegand and S. Pflugmacher (2008). Cyanobacterial toxin elimination via bioaccumulation of MC-LR in aquatic macrophytes: an application of the “green liver concept”. *Environmental Science and Technology* 42: 8552-8557.

- Oliva-Teles L., E. Pereira, M.L. Saker. and V.M. Vasconcelos (2008). Virtual experimentation on cyanobacterial bloom dynamics and its application to a temperate reservoir (Torrao, Portugal). *Lakes & Reservoirs: Research & Management* 13(2):135-143.
- Osswald, J., S. Rellán, A. Gago and V. Vasconcelos (2007). Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. *Environmental International* 33: 1070-1089.
- Osswald, J., S. Rellán, A. Gago and V. Vasconcelos (2009). Production of anatoxin-a by cyanobacterial strains isolated from Portuguese fresh water systems. *Ecotoxicology* 18:1110–1115.
- Ouahid, Y., G. Pérez-Silva and F.F. del Campo (2005). Identification of potentially toxic environmental *Microcystis* by individual and multiple PCR amplification of specific microcystin synthetase gene regions. *Environmental Toxicology* 20:235-242.
- Ouahid Y. 2008. Short and long-term evolution of cyanobacteria biomass and its microcystin content in the drinking-water supply Pinilla. Identification and characterization of microcystin producing and non-producing *Microcystis* strains. *PhD thesis*. Universidade Autónoma de Madrid - Faculdade de Ciências, Madrid, Spain.
- Papadimitriou T, I. Kagalou, C. Stalikas, G. Pilidis and I.D. Leonardos (2012). Assessment of microcystin distribution and biomagnification in tissues of aquatic food web compartments from a shallow lake and evaluation of potential risks to public health. *Ecotoxicology* 21: 1155-1166.
- Pearson, L.A. and B.A. Neilan (2008). The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public risk health. *Current Opinion in Biotechnology* 19: 281-288.
- Pearson, L., T. Mihali, M. Moffitt, R. Kellmann and B.A. Neilan (2010). On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Marine Drugs* 8: 1650-1680.
- Perreault, F., M.S. Matias, S.P. Melegari, C.R.S.C. Pinto, E.E. Creppy, R. Popovic and W.G. Matias (2011). Investigation of animal and algal bioassays for reliable saxitoxin ecotoxicity and cytotoxicity risk evaluation. *Ecotoxicology and Environmental Safety* 74: 1021-1026.
- Poniedziakłek, B., P. Rzymysky and M. Kokociński (2012). Cylindrospermopsin: Water-linked potential threat to human health in Europe. *Environmental Toxicology and Pharmacology* 34(3): 651-660.

- Porteiro, J., H. Calado, M. Pereira, J. Ventura and L. Paramio (2005). Planeamento biofísico e gestão de ecossistemas lacustres: as lagoas dos Açores. Universidade dos Açores. Ponta Delgada.
- Quesada, A., D. Sanchis and D. Carrasco (2004). Cyanobacteria in Spanish reservoirs. How frequently are they toxic? *Limnetica* 23: 109-118.
- Rantala-Ylinen, A., S. Känä, H. Wang, L. Rouhiainen, M. Wahlsten, E. Rizzi, K. Berg, M. Gugger and K. Sivonnen (2011). Anatoxin-a synthetase gene cluster of the cyanobacterium *Anabaena* sp. strain 37 and molecular methods to detect potential producers. *Applied and Environmental Microbiology* 77(20): 7271-7278.
- Rapala J., K. Erkomaa, J. Kukkonen, K. Sivonen and K. Lahti (2002). Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography-UV detection and enzyme-linked immunosorbent assay: Comparison of methods. *Analytica Chimica Acta* 466(2):213-231.
- Regueiras, A. (2009). Comparação de métodos de identificação e quantificação de cianobactérias e suas toxinas na Albufeira do Torrão (Rio Tâmega). *Dissertação de Mestrado em Contaminação e Toxicologia Ambientais*. Universidade do Porto, Porto, Portugal, 91pp.
- Rivasseau C., P. Racaud, A. Deguin and M-C Hennion (1999). Evaluation of an ELISA kit for the monitoring of microcystins (cyanobacterial toxins) in water and algae environmental samples. *Environmental Science & Technology* 33(9):1520-1527.
- Saker, M.L., J.S. Metcalf, G.A. Codd and V.M. Vasconcelos (2004). Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anadonta cygnea*. *Toxicon* 43(2): 185-194.
- Saker M.L., M. Vale, D. Kramer and V.M. Vasconcelos. (2007). Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. *Applied Microbiology and Biotechnology* 75(2):441-449.
- Sangolkar, L.N., S.S. Maske and T. Chakrabarti (2006). Methods for determining microcystins (peptide hepatotoxins) and microcystin-producing cyanobacteria. *Water Research* 40: 3485-3496.
- Schembri M.A., B.A. Neilan and C.P. Saint (2001). Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 16(5):413-421.
- Shen X., P.K.S Lam, G.R. Shaw and W. Wickramasinghe (2002). Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. *Toxicon* 40(10):1499-1501.

- Sivonen, K. and G. Jones (1999). Cyanobacteria toxins *in*: Chorus, I. and J. Bartram (Eds.). *Toxic Cyanobacteria in water: A guide to their public health consequences, monitoring and management*. WHO. E&FN Spon, London.
- Summerfield, T.C. and L.A. Sherman (2008). Global Transcriptional Response of the Alkali-Tolerant Cyanobacterium *Synechocystis* sp. Strain PCC 6803 to a pH 10 Environment. *Applied and Environmental Microbiology* 74: 5276–5284.
- Sze, P. (1993). *A Biology of the Algae 2nd Edition*. William C Brown Pub, USA, 272 pp.
- Teixeira, M.R. and M.J. Rosa (2006). Neurotoxic and hepatotoxic cyanotoxins removal by nanofiltration. *Water Research* 40: 2837-2846.
- Torres, R., E. Pereira, V. Vasconcelos and L.Oliva-Teles (2011). Forecasting of cyanobacterial density in Torrão reservoir using artificial neural networks. *Journal of Environmental Monitoring* 13: 1761-1767.
- Vale M. (2005). Comunidades planctónicas da albufeira do Torrão. Eutrofização e biodiversidade. *Dissertação de mestrado em Hidrobiologia*. Universidade do Porto, Porto, Portugal.
- Van Apeldoorn, M.E., H.P. van Egmond, G.J.A. Speijers and G. J. I. Bakker (2007). Toxins of cyanobacteria. *Molecular Nutrition & Food Research* 51: 7-60.
- Vasconcelos V.M. (1993). Toxicity of cyanobacteria in lakes of north and central Portugal. Ecological implications. *International Verein. Limnology*, 25:694-697.
- Vasconcelos V.M. (1994). Toxic cyanobacteria (blue-green algae) in Portuguese fresh waters. *Archiv für Hydrobiologie* 130(4):439-451.
- Vasconcelos V.M. (1995). Toxicologia de cianobactérias. Distribuição de cianobactérias tóxicas e suas toxinas em águas doces portuguesas. Bioacumulação em bivalves. *Dissertação para a obtenção do grau de Doutor em Biologia*. Faculdade de Ciências da Universidade do Porto, Porto, Portugal, 137pp.
- Vasconcelos, V.M. (1999). Cyanobacterial toxins in Portugal: effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research* 32: 249-254.
- Vasconcelos VM. (2001). Cyanobacteria toxins: Diversity and ecological effects. *Limnetica* 20(1):45-58.
- Vasconcelos VM., K. Sivonen, W.R. Evans, W.W. Carmichael, M. Namikoshi (1995). Isolation and characterization of microcystins (heptapeptide hepatotoxins) from portuguese strains of *Microcystis aeruginosa* Kutz. emend Elekin. *Archiv für Hydrobiologie* 134(3):295-305

- Vasconcelos, V., K. Sinoven, W.R. Evans, W.W. Carmichael and M. Namikoshi (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 35: 1354-1357.
- Washington State Department of Health (2008). Washington State Provisional Recreational Guidance for Microcystin and Anatoxin-a. Final report. Washington, USA, 19 pp.
- Washington State Department of Health (2011). Washington State Provisional Recreational Guidance for Cylindrospermopsin and Saxitoxin. Final report. Washington, USA, 36 pp.
- White, S.H., L.J. Duivenvoorden, L.D. Fabbro and G.K. Eaglesham (2006). Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). *Toxicon* 47(5): 497-509.
- Xie, L., P. Xie, L. Guo, L. Li, Y. Miyabara and H.D. Park (2005). Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu, China. *Environmental Toxicology* 20: 293-300.
- Yavasoglu, A., M.A. Karaaslan, Y. Uyanikgil, F. Sayim, U. Ates and N.U.K. Yavasoglu (2008). Toxic effects of anatoxin-a on tests and sperm counts of male mice. *Experimental and Toxicologic Pathology* 60: 391-396.
- Žegura, B., G. Gajski, A. Štraser and V. Garaj-Vrhovac (2011). Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress. *Toxicon* 58(6-7): 471-479.
- Zhang, D., P. Xie, Y. Liu and T. Qiu (2009). Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. *Science of the Total Environment* 407: 2191-2199.